2003

Dechlorination of 1,2,3,4-Tetrachlorobenzene in organic matter- and mineral-dominated soils and effects of Typha latifolia roots

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DECHLORINATION OF 1,2,3,4-TETRACHLOROBENZENE IN ORGANIC MATTER- AND MINERAL-DOMINATED SOILS AND EFFECTS OF *TYPHA LATIFOLIA* ROOTS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Civil Engineering

in

The Department of Civil and Environmental Engineering

by
Lizhu Lin
B. S., Xiamen University, China, 2000
May 2003

ACKNOWLEDGMENTS

The author would like to express her profound thanks to her major professor, Dr. John H. Pardue, for his constructive guidance, grateful help and support throughout the course of this thesis. Special thanks are also due to Dr. William M. Moe and Dr. Clinton S. Willson for their sincere advice, suggestion and help during the course of the research.

The author wants to extend her grateful appreciation to colleagues, Dr. Gabriel R. Kassenga, Ms. Kimberly Bowman and Ms. Congna Li, for their sincere assistance in conducting the experiments. The author would also like to thank other colleagues of the Wetland Research Group, Dr. Cesar Gomez, Dr. Sangjin Lee, Julius Enock, Ms Eun-Ju Lee, Jason House, Stephen Mbuligwe, and Jeff Maynor, for their help in various ways and sharing experience.

Grateful support of the Hazardous Substance Research Centers/South & Southwest Center (HSRC) for this research project is also highly acknowledged.

Most importantly, the author wishes to express special thanks to her husband, Yunzhou Chai, for his endless love and encouragement; to her parents, Yinmou Lin and Fengyu Lin, and her brother and sister for their constant encouragement and support.

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ABSTRACT

A variety of environments are contaminated with chlorinated benzenes. Therefore, investigating the biodegradation of chlorobenzenes in different types of soils is useful in assessing the feasibility of bioremediation. One mineral-dominated soil: PPI (Petro Processors Inc. site) soil, and three organic matter-dominated soils (natural wetland soil, constructed wetland soil (a mixture of peat, compost and sand), and river sediment) were used to investigate anaerobic biodegradation of 1,2,3,4-Tetrachlorobenzene (1,2,3,4-TeCB) using laboratory microcosms. To determine whether methanogens were directly responsible for dechlorination, a comparative study using 2-bromoethanesulfonic acid (BES) for inhibition of methanogenesis was conducted. Hydrogen and methane concentrations, and microbial diversities were analyzed. The results of the present study showed that 1,2,3,4-TeCB was completely biodegraded in all test soils with different microbial communities. The most dominant dechlorination pathway was: 1,2,3,4-TeCB → 1,2,3-TCB → 1,2-DCB + 1,4-DCB + 1,3-DCB → monochlorobenezene + benzene. The test chemical was biodegraded at rates ranging from 0.023 day^{-1} (half-life time of 30.5 days) to 1.108 day^{-1} (half-life time of 0.6 days), with lag periods varied between 1 and 72 days. Dechlorination kinetics of chlorobenzenes was found to depend on many factors other than organic carbon content. DGGE banding profile, methane concentration and dechlorination activities suggest that BES probably changed the compositions of bacteria consortia, and partly inhibited methanogenesis and chlorobenzene dechlorination. Moreover, methanogens were probably not directly responsible for dechlorination of chlorobenzenes.

Rhizosphere of some plants can enhance rhizodegradation of organic contaminants. Thus, the present study also investigated the effects of *Typha latifolia L.* roots on anaerobic degradation of 1,2,3,4-TeCB. Biodegradation rate constant of 1,2,3,4-TeCB was observed to increase with increasing amounts of roots, indicating that *Typha*, a native wetland plant, could be a very promising vegetation for application in phytoremediation. Due to root matter, higher concentrations of organic acids and hydrogen were observed in treatments with roots compared with the treatment without roots, which probably caused higher dechlorination activities in root-amended microcosms.

CHAPTER 1. INTRODUCTION

1.1 Justification of the Study

Chlorinated benzenes constitute one of the major classes of pollutants in the environment because of their extensive application by industry, which ranges from solvents, odorizers, insect repellents, and fungicides to intermediates in the manufacturing of various chemicals such as the synthesis of some dyes and pesticides (Oliver et al., 1982; Middeldorp et al., 1997). Chlorobenzenes have been introduced into the environment through accidental spillage, leakage of storage facilities, indiscriminate usages and poor disposal practices. Due to their hydrophobic nature and strong persistence, chlorobenzenes have been found in surface waters, groundwater, sediments, soils, sewage sludge, and in the subsurface environment (Schwarzenbach, et al., 1979; Oliver et al., 1982; Bailey, 1983; Pesticide Residue Monitoring Database Users' Manual, FDA website, 2002). Furthermore, chlorobenzenes have the tendency of bioaccumulating in the food chain and, thus, have been found in animals and plant tissues (Adrian et al., 1998; Pavlostathis and Prytula, 2000). According to the US National Primary Drinking Water Regulations, the maximum contaminant level (MCL) for protection of human health and the environment for benzene, 1,2-dichlorobenzene (1,2-DCB), 1,4-dichlorobenzene (1,4-DCB), 1,2,4-trichlorobenzene (1,2,4-TCB) and hexachlorobenzene (HCB) are 0.0, 0.6, 0.075, 0.07, 0.001 mg/L, respectively (EPA, 2002a).

Due to the toxicity and bioaccumulative properties of chlorobenzenes, appropriate methods for remediation of sites contaminated with these pollutants have been investigated by many researchers. A variety of technologies for remediation of chlorinated contaminants are available, including *ex-situ* physical/chemical treatment

such as pump and treat systems, where treatment is provided by air stripping, air sparging, catalytic oxidation, among others; and biological treatment methods such as intrinsic natural attenuation, biostimulation and *ex-situ* bioremediation, e.g. constructed wetland systems (Fetter, 1993). Biological treatment methods are especially suitable for effective renovation of soil and groundwater contaminated with low concentrations of dissolved chlorinated organics, since they involve destruction of contaminants and thus, little to no residual treatment is required, unlike the physical/chemical treatment methods (Fetter, 1993). Furthermore, biological treatment methods are typically implemented at low cost. Therefore, biological methods are more attractive and promising alternatives to traditional physical/chemical methods in remediation of sites contaminated with chlorobenzenes. For these reasons, many laboratory and field studies on biological transformation of chlorobenzenes under aerobic and anaerobic conditions have been conducted to investigate the feasibility of bioremediation in cleaning-up of contaminated sites (Beurskens et al., 1994; Masunaga et al., 1996; Potrawfke et al., 1998).

Chlorobenzenes may be transformed under aerobic and anaerobic conditions. The lower chlorinated benzenes are highly reduced and thus more amenable to oxidative degradation than anaerobic degradation. Organisms that catalyze aerobic degradation have been isolated and studied in pure cultures (Reineke et al., 1984; Debont et al., 1986; Schraa, et al. 1986; Spain, et al., 1987; Sander, et al., 1991; Potrawfke et al., 1998). On the other hand, the more highly chlorinated benzenes are highly oxidized and, therefore, tend to resist aerobic degradation. Highly chlorinated benzenes are susceptible to anaerobic reductive dechlorination to less toxic, lower chlorinated benzenes which may be readily aerobically biodegraded (Fathepure et al., 1988; Pardue, 1992; Holliger et al.,

1992; Pardue et al., 1993; Ramand et al., 1993; Masunaga et al., 1996; Chang et al., 1997; Jackson and Pardue, 1998; Pavlostathis and Prytula, 2000; Wu et al., 2002; Chen et al., 2002). Benzene, one of the final products of anaerobic dechlorination of chlorobenzenes is a known human carcinogen (EPA, 2002b). However, it can be effectively degraded aerobically and can also biodegrade under anaerobic conditions (Burland and Edwards, 1999; Deeb and Alvarez-Cohen; 1999).

The degree of anaerobic dechlorination of chlorobenzenes varies depending on dechlorinating microbial consortia as affected by incubation conditions. Isolation of microbes capable of complete dechlorination of chlorobenzenes has therefore been a subject of intensive research recently. However, obtaining pure chlorobenzenes dechlorinating microbial culture has been difficult because of the high toxicity and the low solubility of chlorobenzenes in water (Adrian et al., 1998; Chang et al., 2002). Strain CBDB1, a strict anaerobe, is the only known pure culture capable of reductively dechlorinating 1,2,3-trichlorobezene (1,2,3-TCB), 1,2,4-TCB and all three tetrachlorobenzene isomers (Adrian et al., 2000). Microbial consortia which are capable of anaerobic reductive dechlorination of chlorinated benzenes have a potential for bioremediation of polluted sites, either *in-situ* (e.g., *in-situ* natural attenuation) or *ex-situ* (e.g., in bioreactors). A subsequent aerobic treatment may then lead to the final mineralization of the lower chlorinated benzenes and benzene. A two-stage process combining initial anaerobic reductive dechlorination to less chlorinated benzenes with further aerobic treatment for complete degradation may be the method of choice (Middeldorp et al., 1997). However, it is also possible that chlorobenzenes can be completely biodegraded to nontoxic compounds such as CO_2 under anaerobic conditions

since benzene can be biodegraded anaerobically (Burland and Edwards, 1999; Deeb and Alvarez-Cohen; 1999).

Few studies have been reported on the potential of dechlorination of chlorobenzenes in different types of soils without external addition of electron donors or nutrients. In the present study, 1,2,3,4- tetrachlorobenzene (1,2,3,4-TeCB) was selected as a test chemical, since it is the most hydrophobic chlorobenzene that could be degraded by certain halorespiring organisms such as Strain CBDB1 (Adrian et al., 2000). Therefore, reductive anaerobic dechlorination of 1,2,3,4-TeCB in three different types of organic matter-dominated soils, i.e., natural wetland soil, constructed wetland soil (a synthetic mixture of peat, sand and compost), and river sediment; and one mineral-dominated soil collected from a Superfund site in Baton Rouge, Louisiana (Petro Processors Inc. (PPI)) were investigated. The purpose of this study was to investigate the potential of these soils for dechlorination of 1,2,3,4-TeCB to provide important information for the feasibility assessment of bioremediation of contaminated sediments. Methane, hydrogen and volatile fatty acids associated with dechlorination reactions were also investigated. Since the role of methanogens in dechlorination is not clear, this study also included the comparison of dechlorination activities and microbial populations under non-inhibited and 2-bromoethanesulfonic acid (BES) inhibited conditions.

Rapid microbial degradation rates of organic chemical residues have been observed in vegetated sediments due to the reaction of the plant roots and associated microbial communities (Anderson et al., 1993; Anderson and Walton, 1995; Narayanan et al., 1995; Pardue et al., 1996; EPA, 2000). However, few studies have investigated the role of the rhizosphere of wetland plants in anaerobic degradation of highly chlorinated

benzenes. To evaluate the role of plants in bioremediation of chlorobenzenes contaminated sites, the effects of the root matter on dechlorination activity and microbial community were also covered in this study.

1.2 Objectives

The overall objective of this study was to develop an understanding of the biodegradation kinetics and pathways of 1,2,3,4-TeCB under active and BES-inhibited methanogenesis in organic matter- and mineral-dominated soils. In addition, the role of the rhizosphere of a wetland plant (*Typha latifolia L.*) in dechlorination of 1,2,3,4-TeCB and its effects on the microbial populations were also investigated. The specific objectives were as follows:

(i) To determine the dechlorination kinetics and pathways of 1,2,3,4-TeCB in three different types of organic matter-dominated soils, i.e., natural wetland soil, constructed wetland soil (a synthetic mixture of peat, sand and compost), and river sediment; and in one mineral-dominated soil: PPI soil;

(ii) To establish and compare the diversity of 1,2,3,4-TeCB dechlorination microbial communities in the test soils under uninhibited and BES-inhibited conditions;

(iii) To investigate the effects of acclimation on degradation kinetics and pathways of the test chemical;

(iv) To correlate dechlorination kinetics of 1,2,3,4-TeCB with concentrations of methane and hydrogen in different types of soils; and

(v) To investigate the effects of wetland plant roots on dechlorination kinetics, biodegradation pathways of 1,2,3,4-TeCB, and the diversity of biodegrading microbial populations.

1.3 Organization of the Thesis

In this thesis, Chapter 1 gives a general background of the research including the justification for conducting this study and research objectives. Chapter 2 is a literature review of anaerobic biodegradation of chlorobenzenes. Chapter 3 presents the results of 1,2,3,4-TeCB biodegradation studies in organic matter- and mineral-dominated soils, including degradation kinetics and pathways, and the diversity of microbial populations. The correlation of biodegradation of the test chemical with methane and hydrogen concentrations is also covered in Chapter 3. The effects of the root matter of a wetland plant, *Typha latifolia L.,* on dechlorination of 1,2,3,4-TeCB and microbial consortia in river sediment are addressed in Chapter 4. Finally, Chapter 5 summarizes the major findings and the implications of the study. Some recommendations for future research are also given.

CHAPTER 2. LITERATURE REVIEW

2.1 Chlorobenzenes Degradation Pathways and Kinetics

In many cases, chlorinated benzenes are present in environments where oxygen is not available (e.g., sediments and deep aquifers). Under these circumstances, many studies have shown that anaerobic reductive dechlorination yields lower chlorinated benzenes (Fathepure et al., 1988; Pardue, 1992; Beurskens et al., 1994; Masunaga et al., 1996; Middeldorp et al., 1997; Pavlostathis and Prytula, 2000). Most of the biodegradation studies of chlorobenzenes have been done using HCB; whereas some of the studies were conducted using 1,2,3-TCB or 1,2,4-TCB, and 1,2,3,5-tetrachlorobenzene (1,2,3,5-TeCB) or 1,2,4,5-tetrachlorobenzene (1,2,4,5-TeCB). However, few studies have been reported on anaerobic transformation of 1,2,3,4-TeCB. In addition, at the time of preparation of this thesis, no study has been reported on the effects of wetland plant roots on anaerobic degradation of 1,2,3,4-TeCB. Degradation pathways and kinetics of the most commonly studied chlorobenzene are discussed below.

The most predominant pathway reported for HCB reductive dechlorination is: HCB → pentachlorobenzene (PentaCB) → 1,2,3,5-TeCB → 1,3,5-TCB (Fathepure et al., 1988; Pardue, 1992; Holliger et al., 1992; Beurskens et al., 1994; Masunaga et al., 1996; Middeldorp et al., 1997; Chang et al., 1997; Pavlostathis and Prytula, 2000). However, 1,2,3,5-TeCB and 1,3,5-TCB were not observed during the dechlorination of HCB and PentaCB in a study conducted by Ramanand et al. (1993). Instead, the pathway observed in that study was as follows: HCB and PentaCB → 1,2,3,4-TeCB → 1,2,3-TCB + 1,2,4-TCB → 1,2-DCB + 1,4-DCB → Chlorobenzene; and 1,2,3,5-TeCB was not dechlorinated when added as a single isomer (Ramanand et al., 1993). A similar

dechlorination pathway of HCB was also reported by Nowak et al. (1996) for enrichments from Saale river sediment. Another possible dechlorinating pathway for HCB is: HCB → PentaCB → 1,2,4,5-TeCB → 1,2,4-TCB → 1,3-DCB + 1,4-DCB + 1,2-DCB (Pardue, 1992; Holliger et al., 1992).

The reported lag periods for dechlorination of chlorobenzenes vary significantly, from no lag time or only a few days to up to 3 months due to the differences in experimental conditions (Holliger et al., 1992; Rammand et al., 1993; Nowak et al., 1996; Chang et al., 1997; Adrian et al., 1998; Chen et al., 2002). A wide range of dechlorination rate constants has also been reported. For example, the first order rate constants for HCB and 1,2,3,4-TeCB reported by Masunaga et al. (1996) are 0.0256 d^{-1} and 0.0382 d^{-1} respectively, while those observed by Pavlostathis and Prytula (2000) are 0.282 d^{-1} and 1.455 d^{-1} respectively. These correspond to half lives of HCB and 1,2,3,4-TeCB ranging from 2.5 to 27.1 days and from 0.5 to 18.1 days, respectively.

2.2 Role of Organic Carbon Content in Dechlorination

Since chlorinated compounds are used as electron acceptors during reductive dechlorination, there must be an appropriate source of carbon for microbial growth in order for reductive dehalogenation to occur (Wiedermeir et al., 1999). Naturally occurring organic matter is one of the potential carbon sources of energy for anaerobic microorganisms. Different groups of microorganisms participate in the degradation of dead organic matter to produce volatile fatty acids (such as propionate and formate) and hydrogen (Conrad, 1999), which may serve as electron donors necessary for driving anaerobic reductive dechlorination reactions. Therefore, the rate and perhaps the extent of dechlorination are expected to depend on the organic carbon fraction of soil if other

factors remain constant. A number of studies have demonstrated that dechlorination kinetics are faster in organic carbon rich soils than in soils poor in organic carbon content because microbial activity depends on the availability of organic carbon (Klečka et al., 1990; Lorah et al., 1997; Lorah and Olsen, 1999; Kassenga et al., 2003). Lorah et al. (1997) observed higher dechlorination rates of TCE in freshwater wetland soils rich in organic carbon (f_{oc} = 0.18) than in sand aquifer materials, which could have organic carbon content as low as 0.0001 (Pardue et. al., 1999). Few, if any, study has been reported on the effects of organic carbon content on dechlorination kinetics of chlorobenzenes.

2.3 Electron Donors

Molecular hydrogen and a number of volatile fatty acids (e.g. lactate, propionate and acetate) and alcohols such as ethanol and methanol have been observed to serve as electron donors during dechlorination reactions of chlorobenzenes (Holliger et al., 1992; Nowak et al., 1996; Adrian et al., 1998; Adrian et al., 2000). To determine the effect of electron donors for chlorobenzene dechlorination, a number of studies have been conducted by adding some potential electron donors to the mixed culture (Holliger et al., 1992; Chang et al., 1997; Middeldorp et al., 1997; Adrian et al., 1998; Chen et al., 2002).

In a defined, synthetic mineral medium without any complex additions and with pyruvate as the carbon and energy source, Adrian et al. (1998) found that formate was used as a direct electron donor, but not hydrogen, because the addition of hydrogen did not increase the extent of dechlorination. However, in the pure dechlorinating culture, Strain CBDB1, chlorobenzenes served as electron acceptors and hydrogen was used as an electron donor in the dehalorespiratory process (Adrain et al., 2000). Middeldorp et al.

(1997) reported that 1,2,4-TCB was able to be degraded with hydrogen, lactate, glucose, propionate, ethanol, methanol or acetate added separately as an electron donor in a methanogenic consortium prepared from a mixture of polluted sediments. However, addition of formate as an electron donor did not support dechlorination of 1,2,4-TCB (Middeldorp et al., 1997). On the other hand, Holliger et al. (1992) reported that hydrogen or lactate enhanced 1,2,3-TCB dechlorination to a greater extent than pyruvate or acetate in enrichment cultures originating from percolation columns filled with Rhine River sediment, in which dechlorination of trichlorobenzenes and dichlorobenzenes (TCBs and DCBs) occurred.

From the preceding discussion, it can be inferred that dechlorination activities of chlorobenzenes vary depending on the type of microbial culture involved as affected by both the type of electron donor and the type of medium used during the study. It is obvious that factors that may influence dechlorination reactions of chlorobenzenes are varied and complex. In most cases it is difficult to ascertain whether a given factor directly affects the specific dechlorinating organism or works against other organisms in the dechlorinating consortium as Chen et al. (2002) also observed.

2.4 Role of Methanogens in Dechlorination

BES, a potent methanogenesis inhibitor, has traditionally been used for evaluation of the role of methanogenesis in dechlorination reactions (Nowak et al., 1996; Middeldorp et al., 1997; Adrian et al., 1998; Pavlostathis and Prytula, 2000). Middeldorp et al. (1997) found that methanogenesis was completely inhibited when 5 mM BES was added, but dechlorination was not inhibited. However, in another methanogenic dechlorinating consortium, BES completely inhibited the dechlorination of

chlorobenzenes, which indicates that BES is not a specific inhibitor for methanogenesis as has been proposed, but may also directly inhibit dechlorination (Middeldorp et al., 1997). It was, therefore, hypothesized that the addition of BES probably excluded methanogens or a methanogen-dependent group of bacteria, which indicated that the dechlorinating population probably consisted of BES-sensitive and BES-insensitive bacteria. Those two different dechlorinating groups of bacteria probably performed different dechlorination patterns (Middeldorp et al., 1997). Pavlostathis and Prytula (2000) also reported that methane production was inhibited in BES-amended culture prepared from contaminated estuarine sediment, but sequential reductive dechlorination of HCB occurred at a comparable rate and a pattern similar to that observed in the non-amended culture, which indicated that methanogens were probably not directly responsible for the reductive dechlorination. Adrian et al. (1998) also consistently observed that methanogenesis was successfully eliminated by the addition of 4 mM BES, and the presence of BES resulted in a significant increase in the extent of trichlorobenzene dechlorination. Therefore, the stimulating effect of BES on dechlorination may have partly been due to the elimination of methanogenic bacteria, which compete with dechlorinating bacteria for electron donors (Adrian et al. 1998). However, Nowak et al. (1996) found that dechlorination of three isomers of trichlorobenzenes in BES-amended culture occurred at a significantly slower rate compared to the un-amended culture, although dechlorination of trichlorobenzenes was observed in BES-adapted culture. These results indicated that methanogens are important for the syntrophic associations within the anaerobic food chain (Nowak et al., 1996).

From the above discussion, it can be concluded that the potency of BES on dechlorinating activities varies significantly depending on the type of methanogenic-dechlorinating consortium involved, among other factors.

2.5 Effects of Rhizosphere on Dechlorination

Enhancement of degradation of organic compounds in the root zone is termed "rhizodegradation", or plant-assisted degradation (EPA, 2000). Rapid degradation of chlorinated organics has been observed in the rhizosphere, the region immediately adjacent to plant roots (Anderson and Walton, 1995; Pardue et al., 1996; Jordahl et al., 1997; Lorah et al., 1999). Anderson and Walton (1995) found that degradation of TCE was accelerated in slurries of rhizosphere soils compared to non-vegetated soil. Jordahl et al. (1997) observed higher populations of benzene-, toluene-, and o-xylene-degrading bacteria in the rhizosphere of poplar trees than in the non-rhizosphere soil.

Enhancement of biodegradation in the rhizosphere could be obtained from the mutual benefit of the interaction between plant roots and microbial communities in the rhizosphere (Anderson et al., 1993). Plants sustain large microbial populations in the rhizosphere by secreting substances such as carbohydrates and amino acids through the root cells and by sloughing root epidermal cells (Anderson et al., 1993). Mucigel (a gelatinous substance secreted by the root cells as a lubricant for root penetration) along with other cell secretions such as organic acids, fatty acids, and amino acids, constitutes root exudates. In the rhizosphere, microbial populations may be nourished from root exudation and decaying plant matter (Anderson et al., 1993). Therefore, the presence of root exudates could contribute to the increase of microbial populations and activities in the rhizosphere (Anderson et al., 1993; and EPA, 2000). Higher microbial counts were

found in the rhizospheres of pesticide-treated plants, which implied that the increase in microbial biomass caused the decrease in persistence of certain toxicants in the rhizosphere (Anderson et al., 1993).

Increased biodegradation rates observed in the rhizosphere may also be the result of greater O_2 concentration provided by the roots (Anderson et al., 1993; Pardue et al., 1999). A zone of aeration is provided when plant roots penetrate the soil (Susarla et al., 2002). Plants transfer oxygen through the leaves and stems to the roots, forming an oxygen layer adjacent to the roots, which may reach a thickness of 0.5 mm (Christensen et al., 1994). Since the rhizosphere has an extremely large surface area, it brings anaerobic (the bulk soil) and aerobic zones in close contact, which may enhance degradation (Pardue et al., 2000).

Another possible factor that could cause accelerated biodegradation in the rhizosphere is that the rhizosphere may provide a habitat in which the microbial consortia capable of growth on organic contaminants may flourish (Anderson et al., 1993). Lappin et al. (1985) found that an individual specie of microorganism isolated from the degrading microbial communities was not capable of growing on or degrading the same herbicide mecoprop used in the same study. However, two or more species together could degrade and grow on mecoprop. Therefore, microbial consortia, rather than individual microbial species, are likely to be involved in the degradation of numerous toxicants in the rhizosphere (Anderson et al., 1993).

2.6 Dechlorinating Organisms

Except for Strain CBDB1 (Adrian et al., 2000), no other bacteria in pure culture capable of dechlorinating chlorobenzenes have been isolated so far. The major limitation

in isolation of chlorobenzene-dechlorinating bacteria is to provide enough chlorobenzene in aqueous phase to sustain growth without the chemical reaching toxic levels (Adrian et al., 1998; Chang et al., 2002). Strain CBDB1 is capable of coupling growth to dechlorination of several CBs including 1,2,3,4-TeCB or 1,2,4,5-TeCB which are degraded to 1,2,4-TCB, and finally 1,3-DCB plus 1,4-DCB; and 1,2,3,5-TeCB which is transformed to 1,3,5-TCB. The isolate does not dechlorinate PentaCB or HCB (Adrian et al., 2000). Recently, Wu et al. (2002) reported the first organism, bacterium DF-1, a polychlorinated biphenyls (PCBs) dechlorinating bacterium, which can dechlorinate chlorobenzenes with more than four chlorines and PCBs.

A number of factors may affect the composition of the dechlorinating population. Temperature is an important factor that may directly affect dechlorinating activities. For example, the optimum temperature for dechlorination found by Chang et al. (1997) was approximately 5°C higher than that reported by Holliger et al. (1992). The differences in the optimum dechlorinating temperature were probably due to the existence of different microbial communities in the two studies among other factors (Chang et al., 1997). Therefore, it is important to correlate dechlorination activities with microbial community, which can be accomplished by using Polymerase Chain Reaction (PCR) based Denaturing Gradient Gel Electrophoresis (DGGE) technique.

It is commonly accepted that PCR-DGGE is a suitable technique to assess the differences in diversities of microbial communities and to monitor changes in microbial consortia (Kozdrój and Elsas, 2000; Casamayor et al., 2000). Using DGGE technique, Chiu and Lee (2001) showed alteration of the bacterial community of an anaerobic enrichment culture that dechlorinated TCE due to long-term exposure to BES.

Macnaughton et al. (1999) were also able to monitor changes in the structure and diversity of the bacterial community during crude oil biodegradation using PCR-DGGE technique. However, the species richness or total microbial diversity in the system can not be accurately estimated with this method (Casamayor et al., 2000). This limitation is due to biases on PCR amplification of DNA (Wintzingerode et al., 1997). In addition, DGGE can not show all populations (Casamayor et al., 2000). Casamayor et al. (2000) reported that populations accounting for less than 1% of the total cell count can not be retrieved by DGGE. Therefore, the image of DGGE fingerprinting patterns provides more information on the structure of the main microbial populations than accurate richness of specific species in the sample.

CHAPTER 3. DECHLORINATION OF 1,2,3,4-TETRACHLOROBENZENE IN ORGANIC MATTER- AND MINERAL-DOMINATED SOILS

3.1 Introduction

Chlorinated benzenes are widespread pollutants and have been found in different environments (Schwarzenbach, et al., 1979; Oliver et al., 1982; Bailey, 1983). Numerous studies have been conducted on the anaerobic reductive dechlorination of chlorobenzenes (Holliger et al., 1992; Pavlostathis and Prytula, 2000), however, few studies have been done on the potential of dechlorination of chlorobenzenes in different types of soils without any addition of electron donors or nutrients. In order to assess the feasibility of natural attenuation and *ex-situ* bioremediation for remediation of chlorinated benzene-contaminated sites, studies on biodegradation of 1,2,3,4-TeCB in different types of soils were conducted. Organic matter-dominated soils (natural wetland soil, constructed wetland soil and river sediment) and mineral-dominated soil (PPI soil) were used to investigate the effects of organic carbon on dechlorination. Since the role of methanogens in dechlorination is yet to be clear, a comparative study using BES as an inhibitor of methanogenesis was conducted. Diversities of microbial communities in the soils were also investigated using DGGE on DNA extracted and amplified from each soil during active dechlorination. Concentrations of methane and hydrogen were measured to establish the correlation between these parameters and dechlorination.

The objectives of the present study were: (i) to determine the dechlorination kinetics and pathways of 1,2,3,4-TeCB in three different types of organic matter-dominated soils, i.e., natural wetland soil, constructed wetland soil, and river sediment;

and in one type of mineral-dominated soil: PPI soil; (ii) to establish and compare the diversities of 1,2,3,4-TeCB dechlorination microbial populations in the test soils and under uninhibited and BES-inhibited conditions; (iii) to investigate the effects of acclimation on degradation kinetics and pathways of the test chemical; and, (iv) to correlate dechlorination kinetics of 1,2,3,4-TeCB with concentrations of methane and hydrogen in the test soils.

3.2 Materials and Methods

3.2.1 Chemicals

Neat 1,2,3,4-TeCB from Supelco (Bellefonte, PA) was used as the test chemical in this study. 1,2,3-TCB, 1,2,4-TCB, 1,3,5-TCB, 1,2-DCB, 1,3-DCB and 1,4-DCB used for calibration were analytical grade from Sigma-Aldrich. Standards for benzene and monochlorobenzene, internal standards and surrogates for EPA Method 8260 were procured from Supelco. Methane used for calibration was also obtained from Supelco Inc. (Supelco, Bellefonte, PA). Hydrogen standards were obtained from BOC Group Inc. (Baton Rouge, LA). HPLC grade hexane and methanol were used as solvents.

3.2.2 Soils

Natural wetland soil was collected from a pristine freshwater wetland in Madisonville, Louisiana. The wetland soil used for microcosm study was a mixture of soils collected from the ground surface to a depth of 30 cm. River sediment was obtained from Bayou Duplantier, Baton Rouge, Louisiana. Constructed wetland soil was a mixture of Bion soil (Dream Maker Dairy, Cowlesville, NY), Latimer peat (Latimer's Peat Moss Farm, West Liberty, OH), and fine to medium sand mixed at a ratio of 1.3: 1.1: 1 (Bion Soil: Latimer peat: Sand) by weight, which was found to be potentially a promising soil

mixture for construction of a treatment wetland for attenuation of chlorinated volatile organic compounds (Kassenga et al., 2003). PPI soil was collected from a former organic wastes disposal site (Petro-Processors Inc. site) in Baton Rouge, Louisiana. The primary organic waste components of the disposal site were hexachlorobutadiene (HCBD) and HCB, halogenated solvents, and polynuclear aromatic hydrocarbons. Trace amounts of chlorobenzenes were detected in the PPI soil used for the present study (data not shown).

Total organic matter (OM) of the test soils was estimated by weighing oven-dried soils before (103 °C for 24 hours) and after combustion at 550 °C for 24 hours (Nyman et al., 1997). Total organic carbon content (TOC) was calculated from total organic matter (OM) using a division factor of 1.7, i.e., TOC = OM /1.7 (Allison, 1965).

3.2.3 Microcosm Experiment

Triplicate anaerobic microcosms were set up in a glove bag (I^2R, Cheltenham, PA) under a nitrogen atmosphere. Four types of test soils, namely, natural wetland soil, constructed wetland soil, river sediment and PPI soil, were homogenized and packed in 160 mL serum bottles leaving 20 mL headspace. A volumetric ratio of water to sediment of 1.5: 1 was used (Lorah et al., 1997). Pore water collected from the freshwater wetland was used for preparation of natural wetland soil microcosms. All other microcosms were prepared using deionized water. All bottles were sealed with Teflon-lined rubber septa and aluminum crimp seals and incubated in an inverted position under static and dark conditions at 25 °C. Microcosms were neither amended with electron donors nor nutritional supplements to support microbial growth.

1,2,3,4-TeCB was dissolved in methanol (Pavlostathis and Prytula, 2000) and then spiked into microcosms to a final concentration of about 150 mg/kg dry weight of

soil. A relatively small volume of methanol of between 0.5 mL and 1 mL (in a total of 140 mL slurry) was used for spiking the test chemical in order to limit the effects of methanol on dechlorination. To minimize the amount of methanol, the bottles were purged with nitrogen at 1 atm using a syringe needle for about 1 min and immediately sealed inside the glove bag. The microbial consortia developed from the first spike of the test chemical into the fresh soil is referred to as the 1st Generation culture. When the concentration of the parent compound dropped below the detection limit (5 ng/μL in hexane extract), 25 mL of slurry from the 1st Generation culture was inoculated into microcosms prepared from fresh soil to develop the 2nd Generation culture. The 3rd Generation culture was developed by inoculating 25 mL of slurry from the 2nd Generation culture. The same experimental conditions were maintained for developing all microbial cultures. Two identical sets of microcosms were set up for each generation culture. One set was used for gas analysis and the other set was used for chlorobenzene analysis and molecular analysis.

To understand the possible role of methanogens in dechlorination of 1,2,3,4-TeCB, treatment using the methanogenesis inhibitor BES was conducted. To account for abiotic losses and to confirm that the disappearance of chlorobenzenes was due to microbial activities, another treatment was prepared using 1% formaldehyde as a biocide. No inoculation was done for abiotic control microcosms. Therefore, three treatments were involved in all test soils, i.e., active control, BES-amended and abiotic control.

For each spike, concentrations of the parent compound and degradation daughter products were monitored until the concentration of the parent compound had dropped below the detection limit of the analytical methods. Sampling of slurry for analysis of

chlorobenzenes was done inside the glove bag (I^2R, Cheltenham, PA) in order to maintain anaerobic conditions in the microcosms. Four mL of soil slurry was withdrawn from microcosms after shaking the bottle to homogenize the contents, the bottle was flushed with nitrogen at 1 atm for about 1 min and resealed. The soil slurry was then transferred into Teflon centrifuge tube to minimize the adsorption of chlorinated benzenes. An equal volume of hexane (i.e., 4 mL) was immediately added into the Teflon centrifuge tubes (Holliger et al., 1992; Chang et al., 1997; Chen et al., 2002). The mixture of slurry and hexane was then tumbled for 24 hours to facilitate the extraction of chlorobenzenes. The suspension was centrifuged at 3,000 rpm for about 15 minutes at room temperature and 1 mL of supernatant was transferred into an amber GC-MS vial. Aqueous samples for analysis of benzene and monochlorobenzene were directly withdrawn from microcosms using a gas tight syringe and transferred to autosampler vials as well. Gas samples for analysis of methane and hydrogen were analyzed without storage.

3.2.4 Analytical Procedures

The hexane extract was analyzed following EPA Method 8270 for the measurement of semivolatile chlorinated benzenes (i.e., tetrachlorobenzenes, trichlorobenzenes and dichlorobenzenes). Ten μL of semivolatile internal standards mix (2000 μg/mL in methylene chloride, containing 1,4-dichlorobenzene-d_4, naphthalene-d_8, acenaphthalene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}) (Supelco Chemical Co.) was injected into 1 mL hexane extract. The sample was then analyzed by GC-MS (Agilent 6890 series gas chromatograph-5972A mass selective detector). The GC was equipped with a capillary column (DB-5, 30 m × 0.25 mm i.d., 0.25 μm film thickness)

which was directly interfaced to the mass spectrometer. High purity helium was used as a carrier gas at a flow rate of 1.8 mL/min. The injector temperature was 250 °C. The GC column was initially held at 37 °C for 2 min, then ramped to 260 °C at 8 °C/min, and finally ramped to 300 °C at 40 °C /min and held for 10 min. The detector temperature was maintained at 280 °C.

Analysis of benzene and chlorobenzene were performed by EPA Method 8260B using a purge and trap apparatus attached to a Agilent 6890 Series Gas Chromatograph equipped with a 5972A mass selective detector. A thermal desorption trap (VOCARB 3000; Supelco, Bellefonte, PA) was employed in the purge and trap apparatus. The hexane extract along with 10 µL internal standard and 2.5 µL surrogate (Supelco, Bellefonte, PA) was manually injected into the purge and trap autosampler (Tekmar 2016) (Tekmar Dohrmann, Mason, OH), and purged for 11 min with high purity helium at a flow rate of 35 mL/min, then desorbed for 0.5 min and baked for 13 min at 225 °C. The samples were then introduced onto the GC equipped with a 30 m × 0.25 mm × 0.25 µm film thickness, Agilent 5MS (5% Phenyl Methyl Siloxane) capillary column (Palo Alto, CA). High purity helium gas was used as a carrier at a flow rate of 2.1 mL/min. The GC column temperature program was -80 °C for 1 min, ramped to 20 °C at 15 °C/min, then ramped to 80 °C at 10 °C/min and finally ramped to 220 °C at 20 °C/min. The temperatures of injector and detector were 250 °C and 280 °C, respectively. Aqueous samples were directly taken from the bottle for the measurement of benzene and chlorobenzene instead of using hexane extraction method. The analytical conditions of GC-MS were the same except that a capillary column with 60 m × 0.32 mm × 3.00 µm film thickness, Agilent 5MS (Palo Alto, CA) was used; and that the GC column

temperature program was 35 °C held for 5 min, then ramped at 4 °C/min to a final temperature of 200 °C.

Prior to sample analysis, six-point calibration curves were established for both methods to determine the relative response factors for the individual compound. Tune, daily blank and calibration check were conducted to assure that the machine and the analytical methods were in control.

Methane was measured by GC-FID. One mL of gas was withdrawn from the headspace of the bottle using a gas tight syringe, and then injected into GC-FID (Agilent 5890 series II) equipped with a 2.4 m × 0.32 mm i. d. column packed with Carbopack b/l % SP-1000 (Supelco, Bellefonte, PA). If methane concentration was found to be higher than the upper range of the linear calibration, the gas sample was diluted using high purity nitrogen at 1 atm. The injector and detector temperatures were 375 °C and 325 °C, respectively. The column temperature was held constant at 50 °C for 6.50 min. High purity nitrogen (BOC Gases, Baton Rouge, LA) was used as a carrier gas at a flow rate of 12 mL/min. All methane data are reported as aqueous concentrations in μM (μmol/L). Headspace methane concentrations were converted to aqueous phase concentrations using Henry's Law (Henry's constant for methane at 25 °C is 0.6364 atm/mol/m^3).

Hydrogen was analyzed using reduction gas analyzer (Trace Analytical, Menlo Park, CA) equipped with a reduction gas detector. Gas samples taken from the headspace were manually injected into a 1-mL gas sampling loop, and then separated with a molecular sieve analytical column (Trace Analytical, Menlo Park, CA) at an oven temperature of 40 °C. The sample was then passed through a catalytical combustion converter (Trace Analytical, Menlo Park, CA) to remove traces of H_2. High purity

nitrogen (BOC Gases, Baton Rouge, LA) was used as a carrier gas. The detection limit under these conditions was 1 ppb. All hydrogen data are reported as aqueous concentration.

Aqueous concentration of H_2 was calculated following the equation adopted from Löffler et al. (1999):

$$[H_{2,aq.}] = LP/RT$$

where $H_{2,aq.}$ is the aqueous concentration of H_2 (moles/L);

L is the Ostwald coefficient for H_2 solubility (0.01913 at 25 °C);

P is the partial pressure of H_2 (atm);

R is the universal gas constant (0.0821 liter·atm·K^{-1}·mol^{-1});

and T is the temperature (K).

$$P = C/10^6$$

where P is the partial pressure of H_2 (atm);

C is the gas phase concentration of H_2 (ppm);

3.2.5 Molecular Analysis

- **DNA Extraction**

Slurry samples were collected from the microcosms, and then immediately stored in sterile cryogenic vials at -20 °C prior to DNA extraction. Extraction of DNA from slurry samples was done following the protocol of Mo Bio Ultraclean Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.) with some modifications. Because of the large amount of humic acids in the soil samples which can inhibit PCR amplification, samples were treated with polyvinylpolypyrrolidone (PVPP) (Agros Organics, Geel, Belgium) (about 0.1 g per 1 g of sample) as a humic acid-binding agent prior to DNA extraction (Holben,

et al., 1988). In order to further remove traces of humic acids, two additional washes using S4 solution (a component of Ultraclean Soil DNA Isolation Kit) were performed. Another modification of the kit protocol was that a Biospec Mini-Beadbeater 3110BX (Biospec products Inc., Bartlesville, OK) was utilized for cell disruption instead of Mo Bio Vortex Adapter (Mo Bio Laboratories, Inc.). The beadbeater was operated at 4,800 rpm for 3.0 min. Extracted DNA was stored at –20 °C until further analysis.

- **PCR Amplification**

Extracted DNA was amplified through Polymerase Chain Reaction (PCR) using an Eppendorf MasterTaq Kit (Brinkmann Instruments, Inc., Westbury, NY). The Eppendorf MasterTaq Kit includes Taq DNA Polymerase (5 U/ μL), 10 × Taq Buffer with Mg^{2+}, and 5 × TaqMaster PCR Enhancer. The 5 × TaqMaster PCR Enhancer often required heating at 60 °C to dissolve the components completely. The master mix was made of 63.5 μL 18 Mega Ohm water, 15 μL of 5 × TaqMaster PCR Enhancer, 10 μL of 10 × Taq Buffer with Mg^{+}, 8 μL of the 10 mM dNTP mix (Applied Biosystems, Forster City, CA), 0.5 μL of the Taq DNA polymerase and 1 μL of each primer (forward and reverse) per sample. For each sample to be amplified, 99 μL of the master mix was placed in a 500-μL sterile PCR reaction tube, and then 1 μL of the extracted DNA was added. This mixture was vortexed and then centrifuged for 1 min at 13,000 rpm. PCR amplification was finally performed by an Eppendorf Thermocycler (Eppendorf GmbH, Hamburg, Germany).

Two different types of primers were applied. One was 341f (5'-CCTACGGGAGGCAGCAG-3') and 907r (5'-CCGTCAATTCMTTTRAGTTT-3') (Casamayor et al., 2000) for the bacteria group; the other set of primers for the archaea

group (i.e., methanogens) was archaeon-specific primers 340f (5'-CCTACGGGGCGCASCAGGSGC-3') and 915r (5'-GTGCTCCCCCGCCAATTCCT-3') (Löffler et al., 1997). An additional 40-nucleotide GC-rich sequence (GC-clamp) attached to the 5' end of both forward primers was: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG (Muyzer et al., 1995). All these primers were obtained from Alpha DNA (Quebec, CA). For the bacteria group, PCR conditions were (Hendrickson et al., 2002): denaturation, 95 °C (2 min); 40 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (1 min) and finally cooling at 4 °C. For the archaea group, the PCR conditions (Löffler et al., 1997) were: denaturation, 94 °C (2 min 10 s); 30 cycles of 94 °C (30 s), 60 °C (45 s), 72 °C (2 min 10 s); final elongation, 72 °C (6 min). PCR products were immediately analyzed or stored at 0 – 4 °C until analysis.

- **Detection of PCR Products**

PCR products were analyzed by the Agilent 2100 Bioanalyzer and corresponding DNA Labchip Kits (Agilent Technology, Willington, DE) to obtain the concentration of DNA and to determine whether the DNA extraction and PCR amplification were successful. One μL of PCR product was used for analysis following the manufacturer's instructions.

- **Denaturing Gradient Gel Electrophoresis (DGGE)**

DGGE was performed using a D-Code™ Universal Mutation Detection System (Bio-Rad, Hercules, CA) as described by Myers et al. (1987) with the following modifications. The 24 mL denaturing gradient gel (6% (wt/vol) acrylamide solution) was covered by a 5 mL acrylamide stacking gel without denaturant. Polymerization was catalyzed with addition of 0.0381% of TEMED (vol/vol) and 0.914% of the 10%

ammonium persulfate (vol/vol) to both denaturant solutions. 0.85% of the 10% ammonium persulfate (vol/vol) and 0.057% of TEMED was added to the 0% stacking gel solution. Gels were cast using a BioRad Model 475 Gradient Delivery System. Samples containing approximately equal amounts of PCR amplicons (with loading dye) were loaded into individual gel lanes. The polyacrylamide gels were made with a denaturing gradient ranging from 40% to 70%, where 100% denaturant contained 42% (wt/vol) urea and 40% (vol/vol) formamide (Bio-Rad, Hercules, CA). Electrophoresis was performed in 1× TAE buffer at 60 °C for 15 hours at 65 V. Following electrophoresis, the gel was stained with ethidium bromide for 10 min. The gel was then destained using 1x TAE buffer for 12 min. Finally, the gel was visualized with a UV transilluminator, photographed and digitized using an Alpha DigiDoc system (Alpha Innotech Co., San Leandro, CA).

3.2.6 Data Analysis

- **Kinetic Data Modeling**

Pseudo first-order kinetic model was applied for modeling kinetic data. To account for abiotic losses of the parent compound in the sterile control, experimental data would be adjusted before the first-order kinetic constant was calculated (Lorah et al., 1997). In the present study, if the percentage recovery rate of the parent compound was higher than 80%, the kinetic data were directly used for modeling. Otherwise, the kinetic data in active control and BES-amended microcosms were modified by comparing with abiotic control microcosms. This kinetic data modification approach assumed that abiotic losses were equal in all treatments for the same type of soil since the experimental

procedures and conditions were the same. Each sampling data was adjusted using the following equation:

$$C_i^{'} = C_i + C_{i-1} \times \left(1 - \frac{C_{Fi}}{C_{Fi-1}} \right)$$

where $C_i^{'}$ is the adjusted concentration for the *i*th sampling point in the active control or BES-amended treatment in a given type of soil (mM/kg dry weight of soil);

C_i is the measured concentration for the *i*th sampling point in the active control or BES-amended treatment in the same type of soil as above (mM/kg dry weight of soil);

C_{i-1} is the measured concentration for the (*i*-1)th sampling point in the active control or BES-amended treatment in the same type of soil as above (mM/kg dry weight of soil);

C_{Fi} is the measured concentration for the *i*th sampling point in the killed control in the same type of soil as above (mM/kg dry weight of soil);

C_{Fi-1} is the measured concentration for the (*i*-1)th sampling point in the killed control in the same type of soil as above (mM/kg dry weight of soil);

First-order reaction rate constant was finally calculated from the first-order kinetic equation shown below by optimization of degradation kinetic data using non-linear regression techniques. When the amount of daughter products detected at the *i*th sampling point was at least 5% of the parent compound after adjustment at the (*i*-1)th

sampling point, the onset of dechlorination was assumed and the lag period was considered to be the time between the *i*th and the (*i*-1)th sampling points.

$$C_t = C_o e^{-kt}$$

where t is the time (day);

C_t is the concentration at any time t (mM/kg dry soil);

C_o is the initial concentration (mM/kg dry soil);

and k is the pseudo first-order reaction rate constant (day^{-1}).

The characteristic half-life period ($t_{1/2}$) was calculated from the first-order reaction rate constant (k) using the following equation:

$$t_{1/2} = -\frac{(\ln 2)}{k} = \frac{0.693}{k}$$

where $t_{1/2}$ is the half-life time (days);

k is the pseudo first-order reaction rate constant (day^{-1}).

- **Statistical Analysis**

Kinetic data were modeled using SigmaPlot 2001. First-order kinetic value and associated standard error were obtained from the non-linear regression analysis. A two-sample t-test was used to compare the differences in first-order kinetic values in different treatments using a significance level of 5%.

3.3 Results and Discussion

3.3.1 Fate of 1,2,3,4-Tetrachlorobenzene in Microcosms

Dechlorination profiles of the 1st Generation cultures of abiotic control and active control microcosms in the four test soils are shown in Figure 3.1 through Figure 3.4. In abiotic control microcosms, the percentage recovery rates of 1,2,3,4-TeCB in the 1st

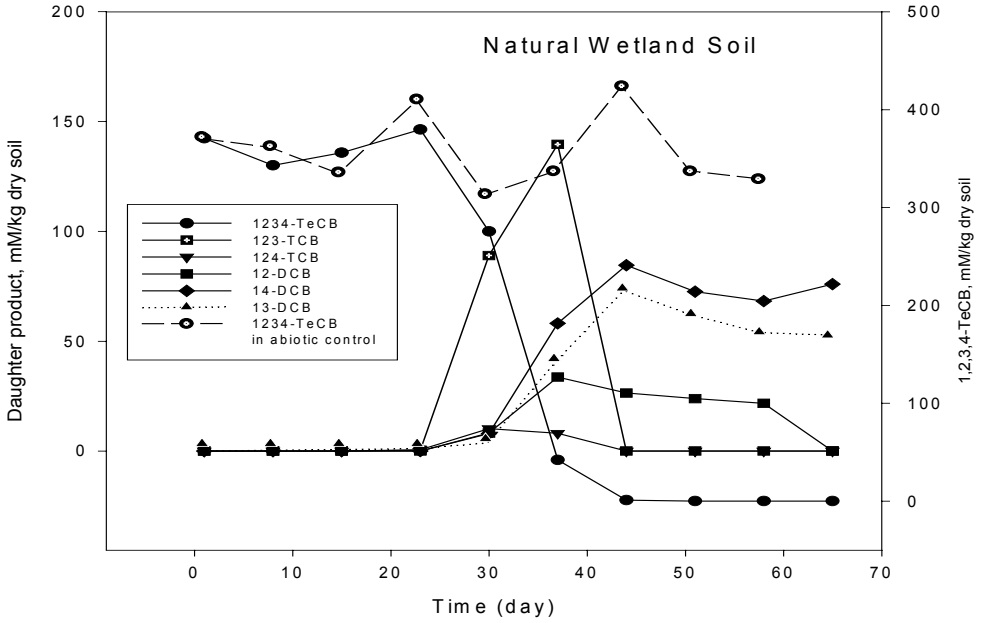

Figure 3.1: 1,2,3,4-TeCB dechlorination profile in the 1st Generation cultures of abiotic and active control microcosms of natural wetland soil.

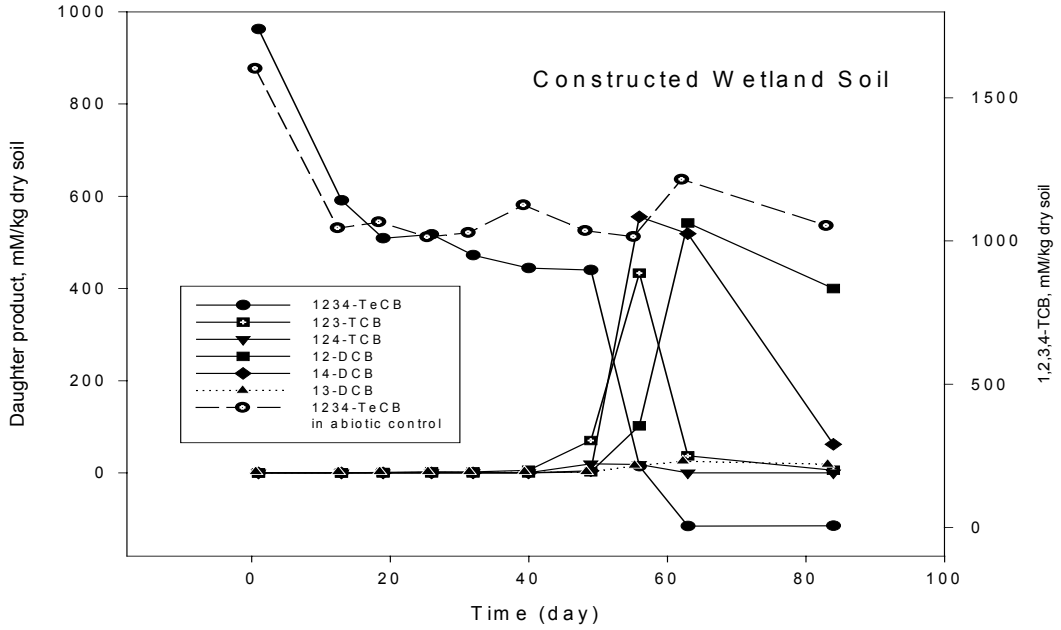

Figure 3.2: 1,2,3,4-TeCB dechlorination profile in the 1st Generation cultures of abiotic and active control microcosms of constructed wetland soil.

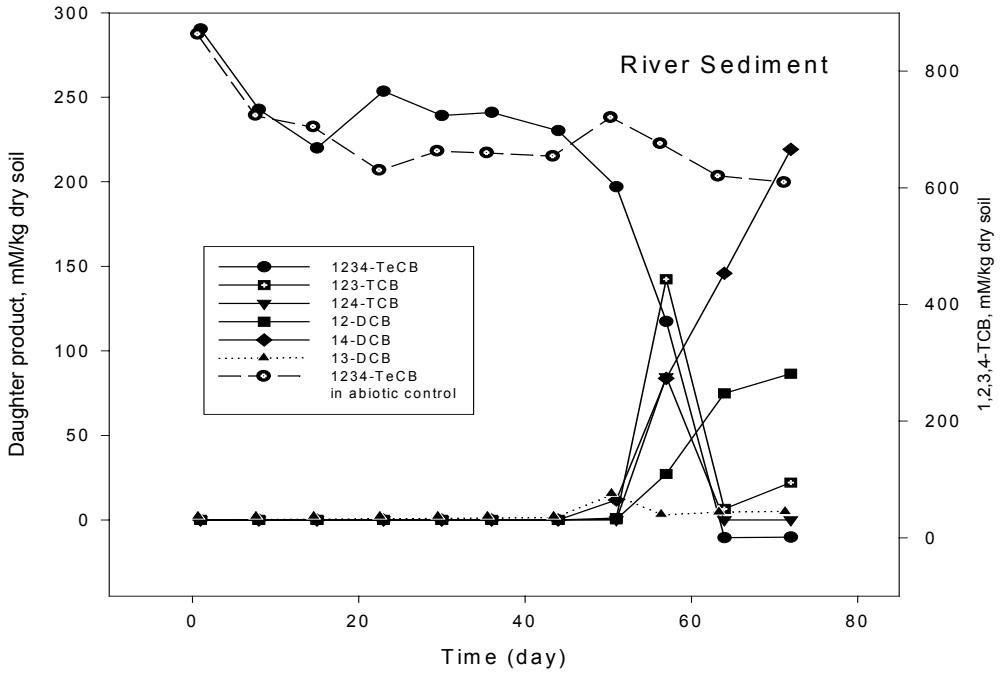

Figure 3.3: 1,2,3,4-TeCB dechlorination profile in the 1st Generation cultures of abiotic and active control microcosms of river sediment.

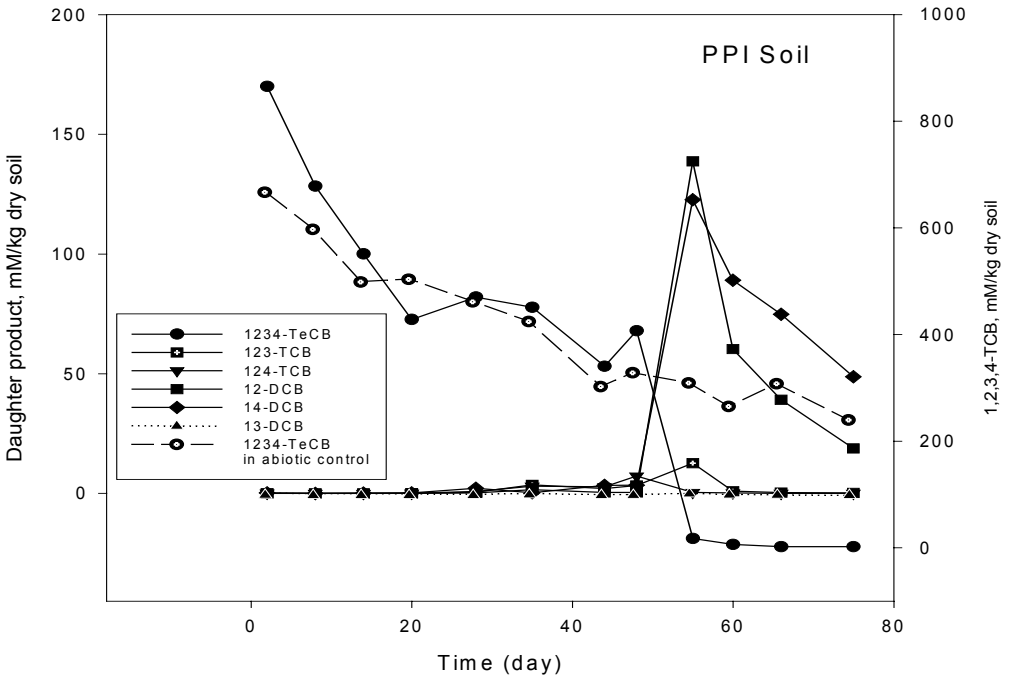

Figure 3.4: 1,2,3,4-TeCB dechlorination profile in the 1st Generation cultures of abiotic and active control microcosms of PPI soil.

Generation cultures of natural wetland soil, constructed wetland soil, river sediment and PPI soil were 87.0 – 107.5%, 92.7 – 111.1%, 70.3 – 100%, and 36.3 – 100%, respectively. Differences in mass balance could be due to experimental errors such as those associated with sampling and analytical procedures. The test compound declined by between 7.3% and 13.0% in abiotic control microcosms of natural wetland soil and constructed wetland soil. This range of losses of the parent compound is very similar to that reported by Ramanand et al. (1993), which ranged from 13.4 to 16.6% for HCB in sodium azide-treated sterile control serum bottles. However, losses of 1,2,3,4-TeCB in abiotic control microcosms of river sediment and PPI soil (up to 63.7%) were higher than those in abiotic control microcosms of natural wetland soil and constructed wetland soil. Similarly, Clover (1998) also observed poor recovery rate as low as 32.2% of the parent compound, monochlorobenzene, in microcosms of PPI soil. The significant differences in the percentage recovery rates of the parent compound could be due to the variations in the extraction efficiencies in different types of soils. In addition, some components acting as strong sorbent such as soot were probably present in the PPI soil, which resulted in low extraction efficiency. Since none of the possible daughter products was detected in all abiotic control microcosms, the low recovery rates of the test chemical were probably caused by abiotic processes rather than microbial activities. On the contrary, significant amounts of daughter products such as trichlorobenzenes and dichlorobenzenes were detected in all active control and BES-amended microcosms as shown in Figure 3.1 through Figure 3.4. Therefore, it can be concluded that the disappearance of 1,2,3,4-TeCB in active control and BES-amended microcosms was due to biodegradation rather than abiotic losses.

3.3.2 Dechlorination Pathways and Kinetics

It was observed that 1,2,3,4-TeCB was completely removed in all microcosms. However, 1,2,3,4-TeCB was biodegraded at different kinetic rates with different lag periods in the test soils. In addition, the kinetic constants and delay times of parent compound dechlorination were significantly different in the 1st, 2nd and 3rd Generation cultures for the same type of soil.

- **Dechlorination Pathways**

Generally, 1,2,3,4-TeCB was biodegraded to 1,2,3-TCB and 1,2,4-TCB; 1,2-DCB, 1,4-DCB and 1,3-DCB; and finally to monochlorobenzene and/or benzene. 1,2,4-TCB and benzene were, however, detected in trace amounts. Dechlorination daughter products in each generation culture for all test soils are listed in Table 3.1. Dechlorination profiles for the 1st, 2nd and 3rd Generation cultures of constructed wetland soil are illustrated in Figure 3.6 (Figure 3.6 A1 through Figure 3.6 A6) as shown later. Dechlorination profiles for natural wetland soil, river sediment and PPI soil are shown in Appendix I, II and III, respectively.

1,2,3-TCB, 1,2-DCB and 1,4-DCB were the major daughter products detected in all test soils (Table 3.1). 1,3-DCB was detected in significant amounts in all generation cultures of active control and BES-amended natural wetland soil microcosms, and the 3rd Generation culture of BES-amended constructed wetland soil microcosms. Otherwise, 1,3-DCB was an insignificant intermediate daughter product in all other microcosms. The degradation pathway observed in the present study is very similar to that reported by Nowak et al. (1996). In the study conducted by Nowak et al. (1996), 1,2,3,4-TeCB was biodegraded to 1,2,3-TCB and all isomers of dichlorobenzenes in 1,3,5-TCB adapted

Table 3.1: List of daughter products in the 1st, 2nd and 3rd Generation cultures of all test soils.

Type of soil		1st Generation	2nd Generation	3rd Generation	Major intermediate products
		Daughter products	Daughter products	Daughter products	
Natural wetland soil	Control	1,2,3-TCB, 1,4-DCB, 1,2-DCB, 1,3-DCB, CB, and Benzene	1,2,3-TCB, 1,4-DCB, 1,2-DCB, 1,3-DCB, CB, and Benzene	1,2,3-TCB, 1,4-DCB, 1,2-DCB, 1,3-DCB, CB, and Benzene	1,2,3-TCB, 1,4-DCB, 1,3-DCB and 1,2-DCB in the 1st, 2nd, and 3rd Generations
	BES	1,2,3-TCB, 1,4-DCB, CB, and Benzene	1,4-DCB, 1,2,3-TCB, CB, and Benzene	1,2,3-TCB, 1,2-DCB, 1,4-DCB, CB, and Benzene	1,2,3-TCB in the 1st Generation; 1,2,3-TCB and 1,4-DCB in the 2nd Generation; 1,2,3-TCB and 1,2-DCB in the 3rd Generation
Constructed soil	Control	1,2,3-TCB, 1,4-DCB, 1,2-DCB, CB	1,2,3-TCB, 1,4-DCB, 1,2-DCB, 1,3-DCB, CB and Benzene	1,2,3-TCB, 1,4-DCB, 1,2-DCB, 1,3-DCB, CB, and Benzene	1,2,3-TCB, 1,4-DCB and 1,2-DCB in the 1st Generation; 1,4-DCB in 2nd Generation; 1,2,3-TCB, 1,4-DCB and 1,2-DCB in the 3rd Generation
	BES	1,2,3-TCB, 1,2-DCB, CB	1,2,3-TCB, 1,2-DCB, 1,4-DCB, CB	1,2,3-TCB, 1,4-DCB, 1,2-DCB, 1,3-DCB, CB, and Benzene	1,2-DCB in the 1st Generation; 1,2,3-TCB and 1,2-DCB in 2nd Generation; 1,2,3-TCB, 1,4-DCB, 1,2-DCB and 1,3-DCB in the 3rd Generation

Table 3.1: (continued)

River sediment	Control	1,2,3-TCB, 1,2-DCB, 1,4-DCB, CB, and Benzene	1,2,3-TCB, 1,2-DCB, 1,4-DCB, 1,3-DCB, CB, and Benzene	1,2,3-TCB, 1,2-DCB, 1,4-DCB, 1,3-DCB, CB, and Benzene	1,2,3-TCB, 1,4-DCB, and 1,2-DCB in the 1st Generation and the same in the 2nd Generation; 1,2,3-TCB and 1,2-DCB in the 3rd Generation
	BES	1,2,3-TCB, 1,2-DCB, 1,4-DCB, CB, and Benzene	1,2,3-TCB, 1,2-DCB, 1,4-DCB, CB, and Benzene	1,2,3-TCB, 1,2-DCB, 1,4-DCB, 1,3-DCB, CB, and Benzene	1,2,3-TCB, 1,4-DCB, and in the 1st Generation; 1,2,3-TCB and 1,2-DCB in the 2nd Generation and the 3rd Generation
PPI soil	Control	1,2,3-TCB, 1,4-DCB, 1,2-DCB, CB, and Benzene	1,2,3-TCB, 1,4-DCB, 1,2-DCB, CB, and Benzene	1,2,3-TCB, 1,2-DCB, 1,3-DCB, CB, and Benzene	1,2-DCB and 1,4-DCB in the 1st Generation; 1,2,3-TCB, 1,2-DCB and 1,4-DCB in the 2nd Generation; 1,2,3-TCB and 1,2-DCB in the 3rd Generation
	BES	1,2,3-TCB, 1,2-DCB, CB, and Benzene	1,2,3-TCB, 1,2-DCB, 1,4-DCB, CB, and Benzene	1,2,3-TCB, 1,2-DCB, 1,4-DCB, 1,3-DCB, CB, and Benzene	1,2,3-TCB and 1,2-DCB in 1st Generation; 1,2-DCB in the 2nd Generation; 1,2,3-TCB and 1,2-DCB in the 3rd Generation

methanogenic consortia. Ramanand et al. (1993) also observed dechlorination of 1,2,3,4-TeCB to 1,2,3-TCB, which was further converted to chlorobenzene via 1,2-DCB. However, the most dominant pathway observed in the present study is significantly different from those reported by Masunaga et al. (1996) and Pavlostathis and Prytula (2000). The authors found that 1,2,3,4-TeCB was mainly dechlorinated to 1,2,4-TCB, and small amounts of 1,2,3-TCB; and 1,4-DCB, 1,3-DCB and 1,2-DCB. Differences in dechlorination patterns of 1,2,3,4-TeCB were probably caused by differences in the compositions of dechlorinating microbial consortia involved in the studies.

Mass balances were calculated to verify that disappearance of the parent compound was due to biodegradation. Since the 2nd and 3rd Generation cultures were developed by inoculating the slurry from the immediate previous generation culture, which contained some daughter products, therefore, mass balance calculations for these generation cultures would not be easily compared. Mass balances for the 1st Generation cultures of active control natural wetland soil and BES-amended river sediment microcosms were found to be 86.3 – 130.2% and 58.7 – 105.2%, respectively. The mass balances obtained in the present study are comparable to the range of 40 – 90% found in the dechlorination study conducted by Masunaga et al. (1996).

Degradation pathways in active control and BES-amended microcosms were slightly different in the same generation for the same type of soil as shown in Table 3.1. In general, the types of major daughter products in those two treatments were different. Moreover, the number of species of the daughter products in BES-amended microcosms was smaller than that in active control microcosms. Middeldorp et al. (1997) also observed different types of daughter products from 1,2,3,4-TeCB dechlorination in BES-

amended and non-amended cultures. These observations indicate that BES most likely changed the microbial community compositions of the test soils and consequently dechlorination pathways of the test chemical.

In the present study, 1,2,3,4-TeCB was observed to be completely transformed in all generation cultures. On the other hand, intermediate degradation daughter products (i.e., 1,2,3-TCB and dichlorobenzenes) were observed to accumulate in all microcosms of the 1st Generation cultures within the incubation periods (Table 3.2). However, complete dechlorination of those intermediate daughter products was observed in all active control microcosms of the 2nd and 3rd Generation cultures, except for the 2nd Generation culture of natural wetland soil. On the other hand, accumulation of the intermediate daughter products (i.e., 1,2,3-TCB and dichlorobenzenes) was found in 50% of BES-amended microcosms of the 2nd and 3rd Generation cultures (Table 3.2). These findings suggest that BES might not inhibit 1,2,3,4-TeCB dechlorination, but rather it may have partially inhibited dechlorination of trichlorobenzenes and dichlorobenzenes. These results are analogous to the observations made by Löffler et al. (1997), who found that dechlorination of tetrachloroethene (PCE) to trichloroethene (TCE) or *cis*-dechloroethene (*cis*-DCE) was not inhibited by BES, whereas dechlorination of DCE isomers and vinyl chloride (VC) was inhibited.

In addition, complete dechlorination of the parent compound and intermediate daughter products (i.e., 1,2,3-TCB and dichlorobenzenes) in all active control microcosms of the 3rd Generation cultures (Table 3.2) indicates that dechlorinating microorganisms were adapted to chlorobenzenes in all organic matter- and mineral-dominated soils. These observations further imply that it is feasible to apply *in-situ* or *ex-*

Table 3.2: Dechlorination statuses of intermediate daughter products (trichlorobenzenes and dichlorobenzenes).

Type of soil		1st Generation	2nd Generation	3rd Generation	Accumulated intermediate daughter products
Natural wetland soil	Control	—	—	+	1,4-DCB, 1,3-DCB and 1,2-DCB in the 1st Generation; 1,4-DCB and 1,3-DCB in the 2nd Generation
	BES	—	—	—	1,2,3-TCB and 1,4-DCB in the 1st Generation; 1,2,3-TCB and 1,4-DCB in the 2nd Generation; 1,2,3-TCB and 1,2-DCB in the 3rd Generation
Constructed soil	Control	—	+	+	1,2-DCB in the 1st Generation
	BES	—	+	—	1,2-DCB in the 1st Generation; 1,4-DCB and 1,3-DCB in the 3rd Generation
River sediment	Control	—	+	+	1,4-DCB and 1,2-DCB in the 1st Generation
	BES	—	—	+	1,2,3-TCB, 1,4-DCB and 1,2-DCB in the 1st Generation; 1,2-DCB in the 2nd Generation
PPI soil	Control	—	+	+	1,4-DCB and traces of 1,2-DCB in the 1st Generation;
	BES	—	+	+	1,2-DCB in 1st Generation; traces of 1,2-DCB in the 2nd Generation, but 1,2-DCB persisted for a relatively long time

+: intermediate degradation daughter products were completely dechlorinated within the incubation periods.
—: intermediate degradation daughter products were accumulated within the incubation periods.

situ bioremediation for cleaning up chlorinated compounds contaminated sites with soils similar in characteristics to the test soils since no accumulation of trichlorobenzenes and dichlorobenzenes is anticipated.

- **Dechlorination Kinetics**

The percentage recovery rates of the parent compound in river sediment and PPI soil were lower than 80%, indicating that abiotic losses of the test chemical were significant. Therefore, pseudo first-order rate constants in river sediment and PPI soil were calculated after adjusting the observed kinetic data to account for the abiotic losses of the test chemical (Lorah et al., 1997). Otherwise, pseudo first-order kinetic constants in natural wetland soil and constructed wetland soil were directly calculated from the measured data since the percentage recovery rates of the parent compound in abiotic control microcosms were higher than 80%.

Kinetic rate constants, half-life times and associated lag periods of dechlorination for all generation cultures in the test soils are shown in Table 3.3. The first-order kinetic model was able to describe degradation kinetics in most microcosms reasonably well as coefficient of determination (R^2) values show (Table 3.3). 1,2,3,4-TeCB was observed to be dechlorinated at significantly different rates in different types of test soils as shown in Table 3.3. Lag periods were also observed to be significantly different in the test soils (Table 3.3).

First-order rate constants ranged between 1.108 day^{-1} and 0.0227 day^{-1}. Corresponding half-life times varied from less than one day to up to 30 days. As for kinetic constants, lag periods also showed a strong variation. In general, lag phases of dechlorination in the 2nd and 3rd Generation cultures were remarkably shorter than those

Table 3.3: Dechlorination rate constants, half-life times and associated lag periods for each generation culture in each type of soil.

Cultures	Natural Wetland Soil				Constructed Soil				River Sediment				PPI Soil			
	Lag period, days	K, day^{-1}	$t_{1/2}$, days	R^2	Lag period, days	K, day^{-1}	$t_{1/2}$, days	R^2	Lag period, days	K, day^{-1}	$t_{1/2}$, days	R^2	Lag period, days	K, day^{-1}	$t_{1/2}$, days	R^2
1st Generation, control	22-29	0.107± 0.026	6.5	0.92	40-49	0.078± 0.029	8.9	0.85	50-57	0.155± 0.027	4.5	0.98	48-55	0.325± 0.009	2.1	0.99
2nd Generation, control	11-14	0.104± 0.022	6.7	0.95	1-8	0.824± 0.079	0.8	0.99	2-12	0.179± 0.006	3.9	0.99	1-5	0.297± 0.016	2.3	0.99
3rd Generation, control	1-5	0.409± 0.019	1.7	1.00	1-5	0.091± 0.019	7.6	0.92	1-6	0.105± 0.010	6.6	0.98	1-5	0.096± 0.029	7.2	0.85
1st Generation, BES	64-72	0.029± 0.010	24.3	0.91	19-26	0.131± 0.030	5.3	0.93	36-44	0.402± 0.015	1.7	0.99	44-48	0.079± 0.01	8.8	0.97
2nd Generation, BES	14-18	0.023± 0.007	30.5	0.82	1-8	0.399± 0.022	1.7	0.99	2-12	0.152± 0.011	4.6	0.99	1-5	0.531± 0.008	1.3	0.99
3rd Generation, BES	1-5	1.108± 0.359	0.6	1.00	1-5	0.190± 0.060	3.7	0.89	1-6	0.073± 0.017	9.5	0.87	1-5	0.135± 0.031	5.1	0.92

K: pseudo first order kinetic constant, day^{-1}; ±: standard error of the pseudo first-order kinetic constant from the non-linear regression; $t_{1/2}$: half-life time, days; R^2: coefficient of determination for the non-linear regression.

in the 1st Generation cultures as shown in Table 3.3. An example of dechlorination profiles of the 1st, 2nd and 3rd Generation cultures is shown Figure 3.5. These observations indicate that dechlorinating microorganisms were able to acclimatize to the test chemical in all test soils. Long lag periods have also been observed in other previous studies, during which microbial populations produced required enzymes for metabolisms of new substrates (Lorah and Olsen, 1999; Etienne et al., 2001).

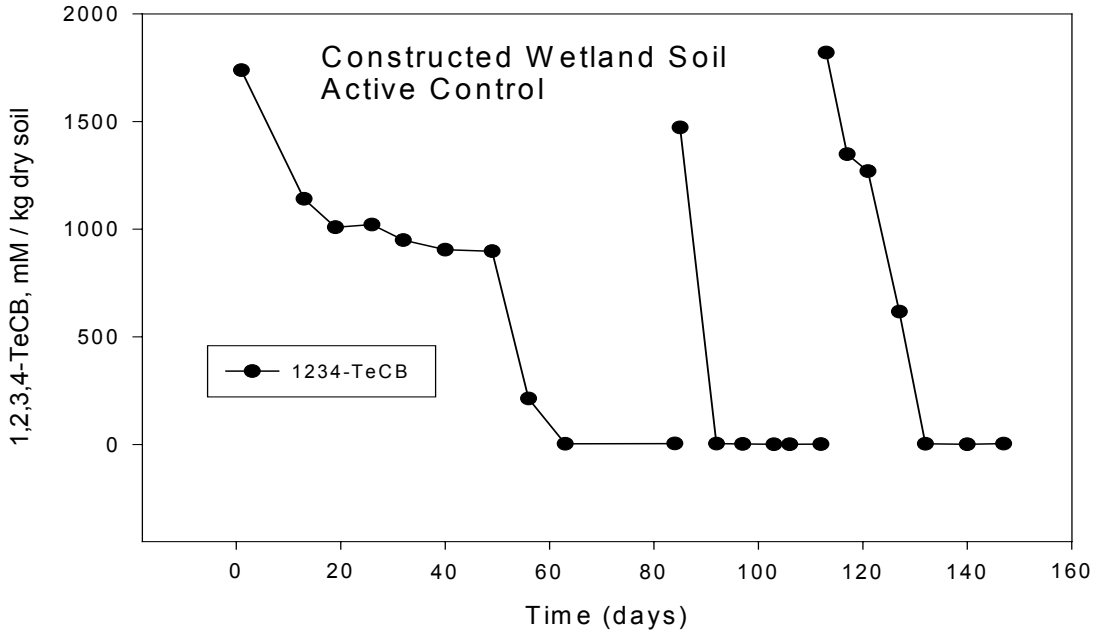

Figure 3.5: 1,2,3,4-TeCB dechlorination profiles in the 1st, 2nd and 3rd Generation cultures of active control constructed wetland soil microcosms.

Lag periods ranged from less than one day to 20 days in the 2nd and 3rd Generation cultures, whereas it took up to about 22 – 72 days for dechlorination to start in the 1st Generation cultures. These findings are similar to those observed by Holliger et al. (1992). In the reported study, much shorter lag period (47 days) of tetrachlorobenzenes dechlorination in the 2nd Generation enrichment cultures was observed than that (73 days)

in the 1st Generation enrichment cultures inoculated with column material, in which dechlorination occurred. Moreover, it can be observed that the 1st Generation culture in the present study had shorter or similar lag phases even without inoculation compared with that in the inoculated 1st Generation enrichment culture in the study conducted by Holliger et al. (1992). Higher dechlorination activities were observed in the current study compared with the findings of Holliger et al. (1992). These results show that natural attenuation for treatment of chlorinated benzenes is feasible even without biostimulation in the test soils.

It was also observed in the present study that longer lag periods were not always associated with lower kinetic constants, which suggests that long delay time of dechlorination does not necessarily indicate a low degradation kinetic rate. For example, the second longest lag period for active control microcosms was between 48 – 55 days in the 1st Generation culture of PPI soil, however, the corresponding first-order kinetic constant ($k = 0.325$ day^{-1}, $t_{1/2} = 2.1$ days) was the highest in the 1st Generation cultures of active control microcosms (Table 3.3). On the other hand, the 1st Generation culture of active control natural wetland soil had the shortest lag period (22-29 days) but had the second lowest kinetic constant (0.107 day^{-1}, $t_{1/2} = 6.5$ days). Therefore, dechlorination kinetic constant and associated lag period are both very important parameters in describing and comparing biodegradation kinetics. Degradation kinetics of the test chemical in different generation cultures are discussed below.

1st Generation Cultures

In the 1st Generation cultures of active control microcosms, the dechlorination rate constant in PPI soil was significantly higher than that in the other three test soils (P >

0.05) as Table 3.3 shows. The total organic carbon contents of the test soils are shown in Table 3.4. According to previous studies, dechlorination rates are generally faster in soils with high organic carbon content compared to soils poor in organic carbon composition (Klečka et al., 1990; Lorah et al., 1997; Lorah and Olsen, 1999; Kassenga et al., 2003). Therefore, based on organic carbon content only, the dechlorination rate constant in PPI soil was expected to be the lowest since it had the lowest organic carbon content (Table 3.4). However, the results obtained in the present study were contrary to what was expected, since degradation kinetics in PPI soil was the highest (Table 3.3). This is probably because PPI soil was previously contaminated by chlorobenzenes (Trace amounts of trichlorobenzenes and dichlorobenzenes were detected in the original PPI soil prior to spiking of the test chemical). Thus indigenous microbial consortia were acclimatized with the test chemical even before the experiments started and thus enhanced the dechlorination of 1,2,3,4-TeCB in spite the fact that it was a mineral-dominated soil poor in organic carbon content. Therefore, degradation rate is affected not only by the organic carbon content but also by other factors, including previous exposure history.

Table 3.4: Total organic carbon contents of the test soils.

Type of soil	Total organic carbon content, % (w/w)	
	Average, %	Standard deviation, %
Natural wetland soil	21.19	0.18
Constructed wetland soil	16.78	2.01
River sediment	6.10	0.13
PPI soil	2.96	0.10

In the 1st Generation cultures of BES-amended microcosms, there were no significant differences in the rate constants between constructed wetland soil and PPI soil ($P > 0.05$) as shown in Table 3.3. The dechlorination rate in river sediment was significantly higher than in the other soils, whereas the dechlorination rate in natural wetland soil was significantly lower than in the other soils ($P > 0.05$) (Table 3.3). On the other hand, there were no significant differences in dechlorination kinetic constants between the active control and corresponding BES-amended microcosms for constructed wetland soil in the 1st Generation cultures (Table 3.3). However, dechlorination rate in BES-amended river sediment was significantly higher than that in the corresponding active control microcosm (Table 3.3), whereas degradation rates in BES-amended natural wetland soil and PPI soil were significantly lower than those in the corresponding active control microcosm (Table 3.3). This suggests that BES had varying effects on the degradation of 1,2,3,4-TeCB in the test soils. These observations are consistent with the findings of other researchers. Pavlostathis and Prytula (2000) found that HCB dechlorination in BES-amended culture occurred at a comparable rate to that observed in non-amended culture. Adrian et al. (1998) observed a significant increase in the extent of trichlorobenzene dechlorination in the presence of BES, whereas a significantly slower rate of dechlorination of three isomers of trichlorobenzenes was observed in BES-amended culture compared with the un-amended culture (Nowak et al., 1996).

2nd Generation Cultures

The degradation kinetics in active control microcosms of the 2nd Generation cultures were observed to be either significantly higher or comparable to those in the 1st Generation cultures ($P > 0.05$) as shown in Table 3.3. Although no significant increase in

rate constants was observed in some microcosms, remarkably shorter lag periods observed (Table 3.3) suggested that the dechlorinating microorganisms were probably acclimatized to the test chemical and supported in those test soils. Based on these findings, natural attenuation or *ex-situ* bioremediation (such as treatment wetlands) could be used for continuous treatment of chlorinated contaminants because the biodegradation rate in the soils increased and lag period decreased with time.

In the 2nd Generation cultures of active control microcosms, comparable lag periods were found in all the test soils. However, the kinetic rate constant in constructed wetland soil was significantly higher than those in the other test soils for the 2nd Generation cultures ($P > 0.05$) as shown in Table 3.3. The corresponding half-life time was 0.8 day, which was much shorter than the time reported in most studies (Holliger et al., 1992; Masunaga et al., 1996). Although the 1st Generation culture of active control constructed wetland soil had the lowest degradation rate, the kinetic rate in the 2nd Generation culture of active control constructed wetland soil was not only much higher than that in its 1st Generation culture, but also significantly higher than those in the 2nd Generation cultures of active control microcosms of the other types of soils. Kassenga (2003) also observed high dechlorination rates of chlorinated aliphatic organic compounds in constructed wetland soil. Therefore, constructed wetland soil could be a very promising material for *ex-situ* bioremediation of chlorinated solvents contaminated sites using treatment wetlands.

In the 2nd Generation cultures of BES-amended microcosms, PPI soil had the highest kinetic rate constant, followed by the constructed wetland soil, river sediment and natural wetland soil (Table 3.3). This order in kinetic rate constant is different from that

found in the 2nd Generation cultures of active control microcosms. Dechlorination rates in active control microcosms of natural wetland soil and constructed wetland soil were significantly higher than those in the corresponding BES-amended microcosms (Table 3.3), indicating that BES probably slowed the dechlorination rate of 1,2,3,4-TeCB although the parent compound was completely degraded. It is also possible that either BES did not specifically inhibit methanogens, or methanogens were not responsible for dechlorination (Middeldorp et al., 1997), as will be discussed later. Nowak et al. (1996) also observed significantly lower degradation rates of trichlorobenzenes in BES-amended mixed cultures than in control cultures. However, in the present study, significantly higher degradation rate in BES-amended microcosms than that in the corresponding active control microcosms was observed in the 2nd Generation culture of PPI soil (Table 3.3). Similar results were obtained by Adrian et al. (1998). The authors speculated that the stimulating effect of BES on dechlorination was probably due to the elimination of methanogens, which competed with dechlorinating microorganisms for electron donors. In the current study, therefore, it is difficult to explain the effects of BES on dechlorination based on the kinetic rates only since conflicting results were obtained in the 1st and 2nd Generation cultures.

3rd Generation Cultures

In the 3rd Generation cultures, the lag periods of dechlorination were either shorter or the same as those in the 2nd Generation cultures, which again indicates that the test soils could support the dechlorinating microorganisms once the organisms were adapted to the substrate. In the 3rd Generation culture of active control and BES-amended natural wetland soil, the dechlorination rate was significantly higher than those in the 1st and 2nd

Generation cultures ($P > 0.05$) as shown in Table 3.3. Shorter lag periods and increasing degradation kinetic rates imply that natural attenuation of chlorinated benzenes is feasible in natural wetlands. Based on laboratory and field studies, Lorah et al. (1997) observed effective attenuation of chloroethenes mainly via biodegradation in natural wetland system. The present study and that of Lorah et al. (1997), therefore, suggest that natural wetlands are naturally capable of degrading chlorinated solvents.

However, the kinetic rate constants in the 3rd Generation cultures of active control microcosms of constructed wetland soil, river sediment and PPI soil were significantly lower than those in the 2nd Generation cultures contrary to expectations (Table 3.3). The unexpected trend of dechlorination kinetics relative to generation cultures was probably caused by differences in characteristics of the soils used for preparing microcosms, in which different generation cultures were developed. Fresh soil collected from the field was immediately used for developing all three generation cultures of natural wetland soil. Therefore, the characteristics of soils in all generation cultures of natural wetland soil were probably the same. However, the river sediment and PPI soil used in the 3rd Generation cultures were stored for about three months before they were used. The chemical characteristics as well as the amount and diversities of the microbial communities most likely changed during the storage period, and thus affected dechlorination activities, because the soils became aerobic in certain portions due to exposure to the air. The color of PPI soil changed from gray to reddish brown possibly because of iron oxidation, implying that chemical characteristics of the soil were altered during the storage period. The observed decrease in kinetic rates for the 3rd Generation cultures of constructed wetland soil was possibly because of the differences in the

characteristics of Bion soil (one of the components of constructed wetland soil) in different batches purchased from the company. Therefore, higher dechlorinating kinetics with associated shorter lag periods would be expected after multiple inoculations if soil characteristics and experimental conditions had remained unchanged, as it was the case for natural wetland soil.

3.3.3 Hydrogen and Methane Concentrations

In the present study, two identical sets of microcosms were set up. One set was used for collecting slurry samples and the other was used for gas analysis. Hydrogen and methane samples were measured at the same time slurry samples were collected in order to correlate dechlorination with hydrogen and methane concentrations. Dechlorination profiles and associated hydrogen and methane concentration trends in constructed wetland soil microcosms are shown in Figure 3.6. Dechlorination profiles and methane and hydrogen concentration trends in the other three test soils are shown in Appendix I, II and III.

In the 1st Generation culture of active control constructed wetland soil microcosms, hydrogen concentration decreased while methane concentration increased before dechlorination started. During the dechlorination period, hydrogen concentration decreased even further (Figure 3.6 A1 and B1). These results indicate that hydrogen was probably used as an electron donor during methanogenesis and for driving dechlorination reactions (Middeldorp et al., 1997; Löffler et al., 1997; Fennell and Gossett, 1998; Adrian et al., 2000). In about 50% of all active control microcosms, hydrogen concentration decreased when dechlorination started and then decreased or remained approximately constant. This trend was observed in the 1st Generation culture of constructed wetland

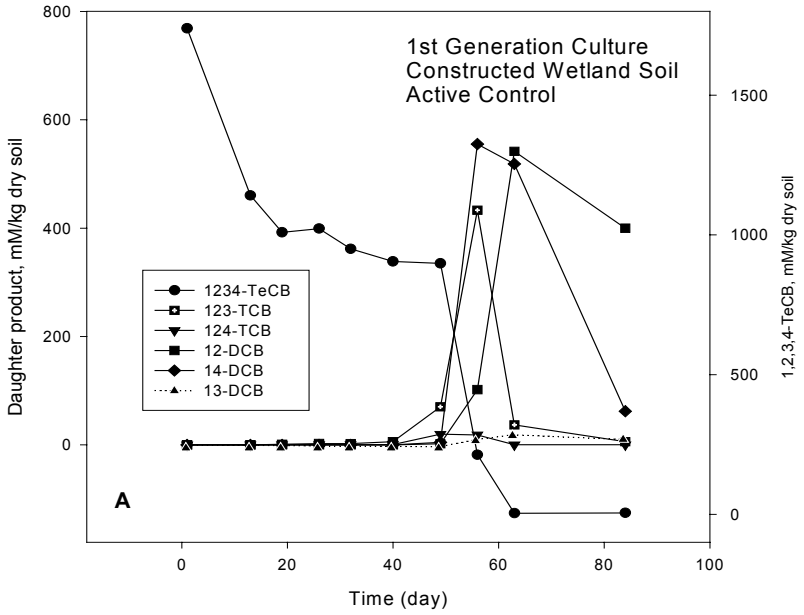

Figure 3.6 A1: Dechlorination profile of the 1st Generation culture of active control constructed wetland soil microcosms.

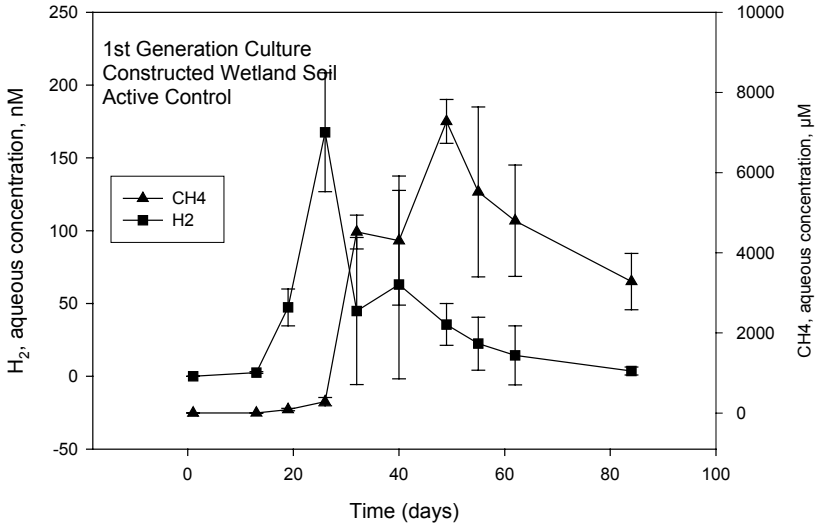

Figure 3.6 B1: Methane and hydrogen concentrations of the 1st Generation culture of active control constructed wetland soil microcosms.

Figure 3.6: Dechlorination profiles and methane and hydrogen concentration trends in constructed wetland soil microcosms. A: Dechlorination profiles; B: Methane and hydrogen concentrations.

Figure 3.6: (continued)

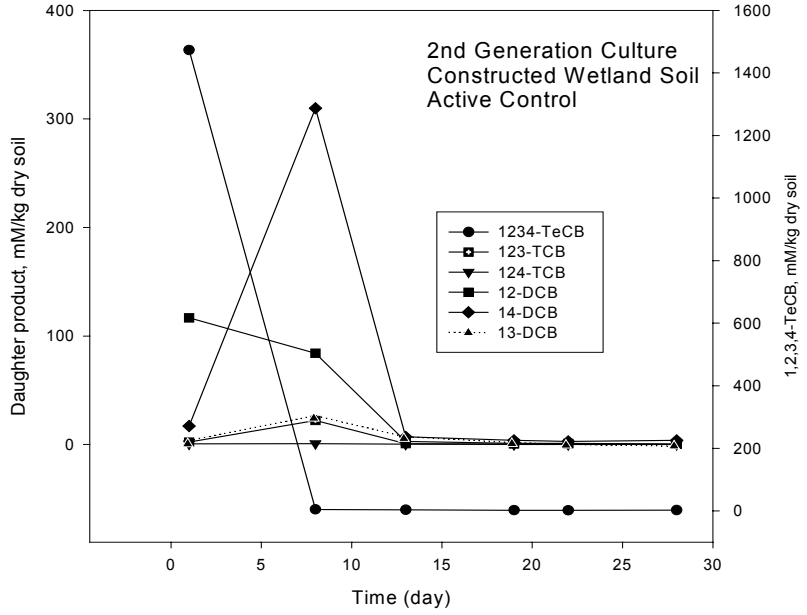

Figure 3.6 A2: Dechlorination profile of the 2nd Generation culture of active control constructed wetland soil microcosms.

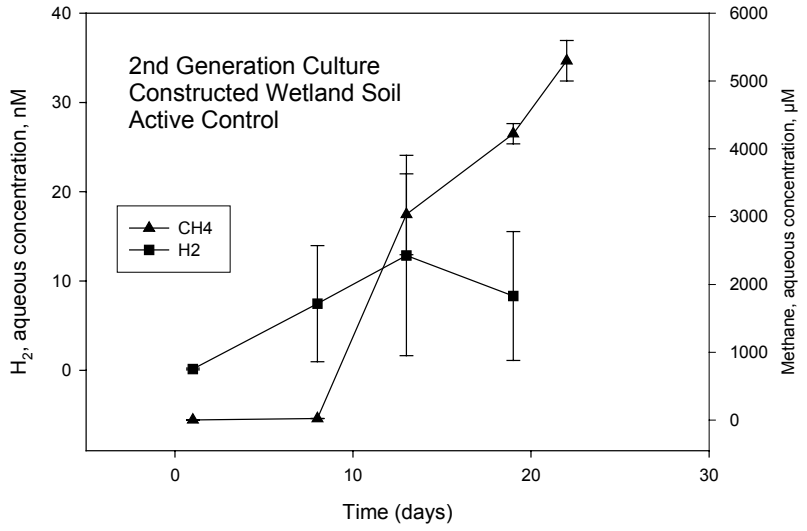

Figure 3.6 B2: Methane and hydrogen concentrations of the 2nd Generation culture of active control constructed wetland soil microcosms

Figure 3.6: (continued)

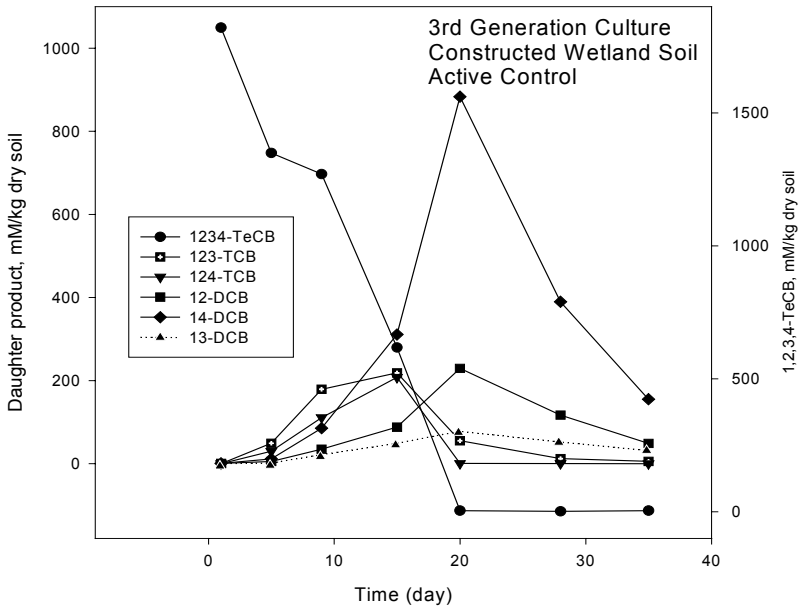

Figure 3.6 A3: Dechlorination profile of the 3rd Generation culture of active control constructed wetland soil microcosms

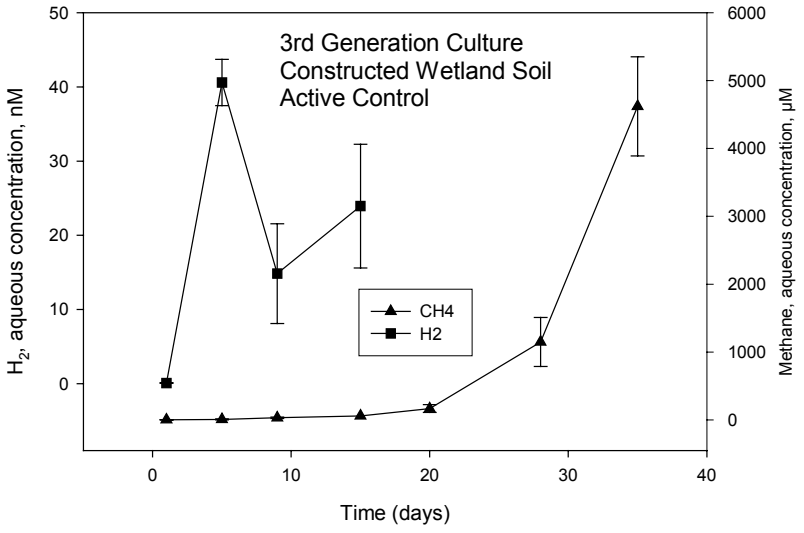

Figure 3.6 B3: Methane and hydrogen concentrations of the 3rd Generation culture of active control constructed wetland soil microcosms

Figure 3.6: (continued)

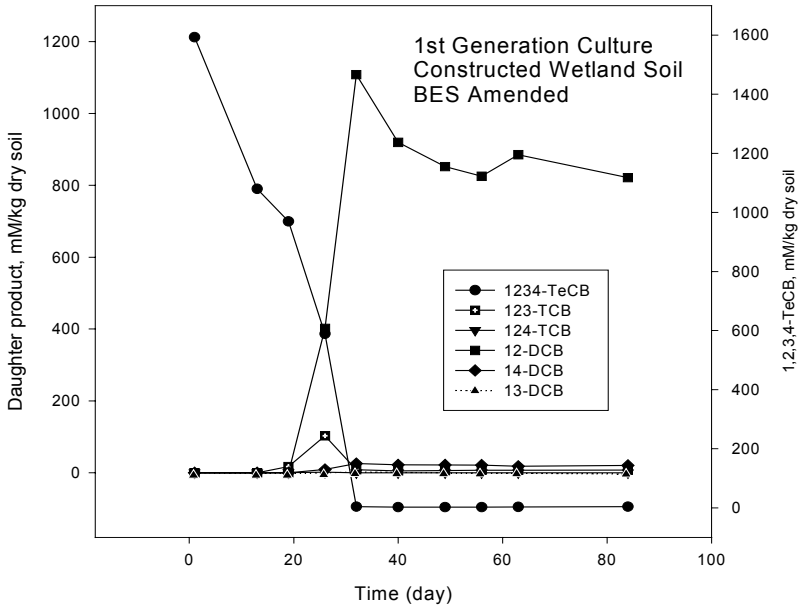

Figure 3.6 A4: Dechlorination profile of the 1st Generation culture of BES-amended constructed wetland soil microcosms

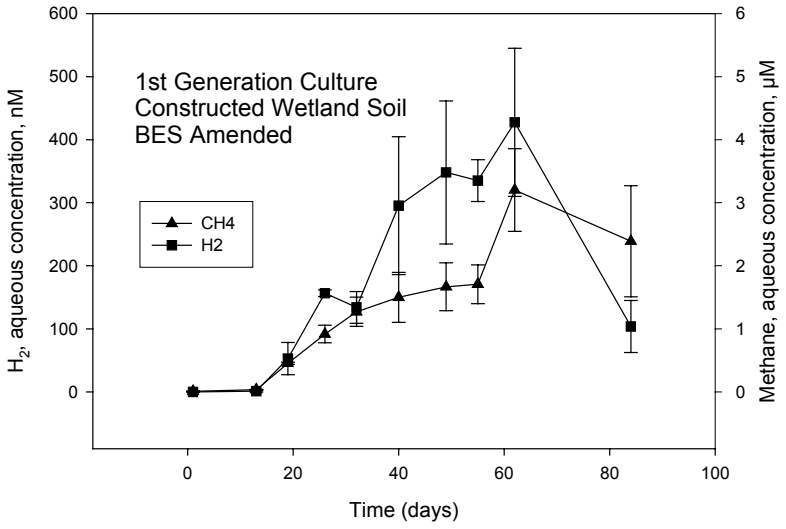

Figure 3.6 B4: Methane and hydrogen concentrations of the 1st Generation culture of BES-amended constructed wetland soil microcosms

Figure 3.6: (continued)

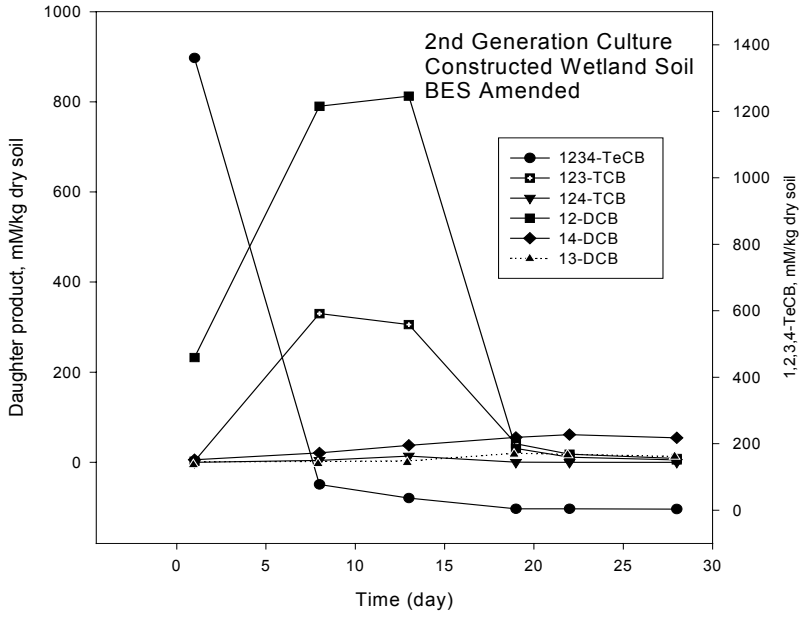

Figure 3.6 A5: Dechlorination profile of the 2nd Generation culture of BES-amended constructed wetland soil microcosms

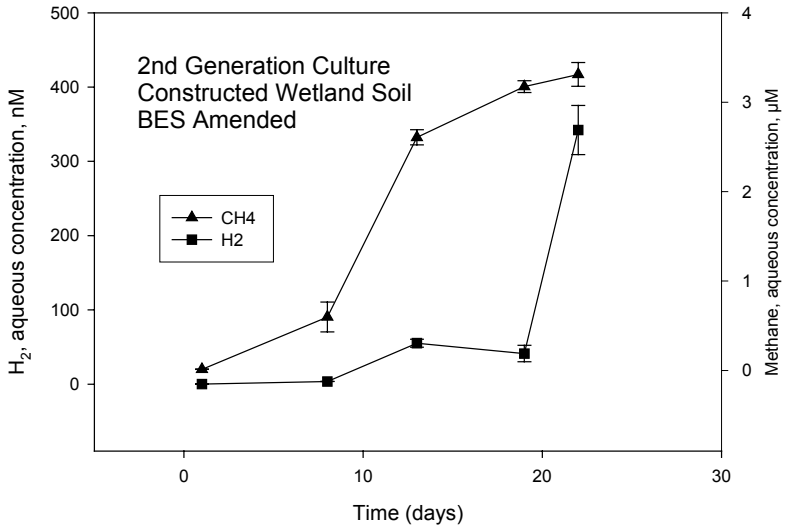

Figure 3.6 B5: Methane and hydrogen concentrations of the 2nd Generation culture of BES-amended constructed wetland soil microcosms

Figure 3.6: (continued)

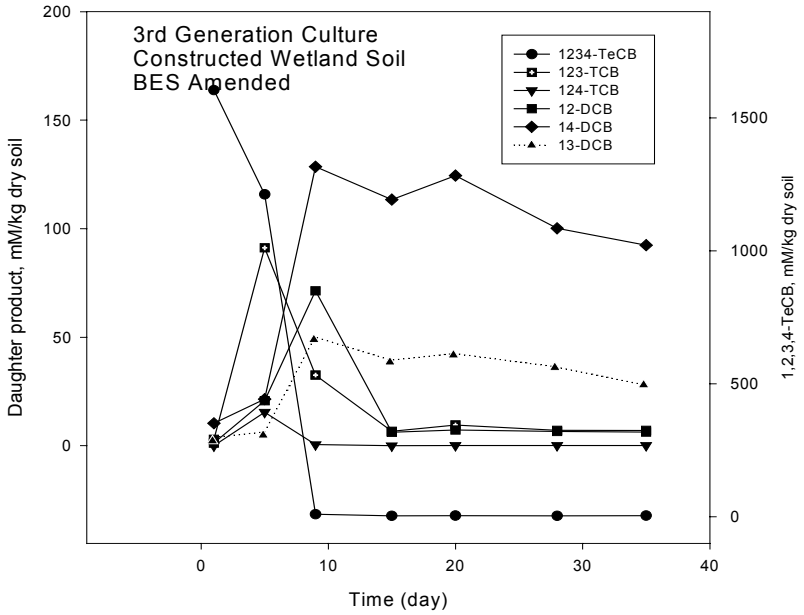

Figure 3.6 A6: Dechlorination profile of the 3rd Generation culture of BES-amended constructed wetland soil microcosms.

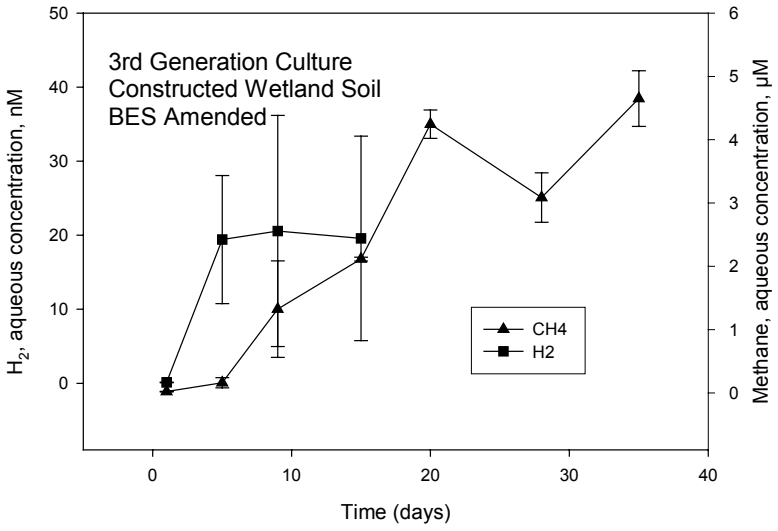

Figure 3.6 B6: Methane and hydrogen concentrations of the 3rd Generation culture of BES-amended constructed wetland soil microcosms.

soil (Figure 3.6 A1 and B1) and the 1st and 3rd Generation cultures of natural wetland soil (Appendix I Figure A1, B1 and A3, B3), river sediment (Appendix II Figure C1, D1 and C3, D3) and PPI soil (Appendix III Figure E1, F1 and E3, F3). However, no clear relationship between hydrogen concentration and dechlorination was found in the other active control microcosms. Generally, it was difficult to correlate 1,2,3,4-TeCB dechlorination with hydrogen concentration levels since hydrogen was produced and consumed by microorganisms at the same time. Moreover, due to insufficient data as a result of unavailability of analytical instruments, correlation of hydrogen concentration with dechlorination was difficult (Figure 3.6 B3 and B6, and Appendix I, II and III).

Dechlorination patterns of the parent compound and daughter products were found to correlate with methane concentration trends in most active control microcosms. However, the relationship between dechlorination profiles and methane concentration trends in BES-amended microcosms could not be clearly defined, probably because the effects of BES on microbial consortia are yet to be clearly understood (Middeldorp et al., 1997; Adrian et al., 1998; Pavlostathis and Prytula, 2000).

- **Active Control**

Except for the 1st and 3rd Generation cultures of active control PPI soil (Appendix III Figure E1, F1 and E3, F3) and the 3rd Generation culture of active control river sediment (Appendix II Figure C3, D3), methane concentrations in all other active control microcosms remained approximately constant or slightly decreased during dechlorination period. In the 2nd and 3rd Generation cultures of constructed wetland soil, and the 2nd Generation cultures of river sediment and PPI soil, methane concentration immediately increased after the parent compound and intermediate daughter products

(trichlorobenzenes and dichlorobenzenes) were completely biodegraded as illustrated in Figure 3.6 A2, B2 and A3, B3, Appendix II (Figure C2 and D2), and Appendix III (Figure E2 and F2). Similar pattern was observed in the 3rd Generation culture of natural wetland soil (Appendix I Figure A3 and B3). Although no clear trends of methane concentration were observed in the 1st Generation culture of PPI soil and the 3rd Generation cultures of river sediment and PPI soil, it can generally be observed that methanogenesis was inhibited during dechlorination periods and methane concentration increased after the parent compound and intermediate daughter products (trichlorobenzenes and dichlorobenzenes) were completely dechlorinated.

There are two possible explanations to the phenomena observed above. The first possibility is that dechlorination of chlorobenzenes was probably mediated by methanogens, which were able to use both chlorobenzenes and carbon dioxide as electron acceptors. Methanogens have been reported to be able to use other chlorinated solvents such as tetrachloroethene and 1,2-dichloroethane as electron acceptors (Tandol et al., 1994; Fantroussi et al., 1998; Klečka et al., 1998). Significantly higher free energy is produced per mole of hydrogen consumed during 1,2,3,4-TeCB dechlorination (-155.2 kJ/mole H_2) (Dolfing and Harrison, 1992) compared to the energy released during CO_2 reduction (-32.7 kJ/mole H_2) (Conrad and Klose, 2000). Since higher energy is yielded when chlorobenzenes are used as electron acceptors compared to carbon dioxide (Dolfing and Harrison, 1992; Conrad and Klose, 2000), methanogens would most likely prefer to use chlorobenzenes to carbon dioxide as energy source when both substrates are present. Therefore, it is possible that methanogens started to use carbon dioxide as an electron acceptor to produce methane after all chlorobenzenes were completely dechlorinated. The

second possibility is that dechlorinating microorganisms out-competed methanogens for hydrogen as an electron donor. During the dechlorination period, the dechlorinating microorganisms probably prevented methanogens from using hydrogen even though the population of potentially active methanogens was relatively higher than that of dechlorinators in the soils. Thus, methanogens were not able to use hydrogen as an electron donor until dechlorination was complete.

In the 1st and 2nd Generation cultures of active control microcosms of natural wetland soil (Appendix I Figure A1 through B2) and the 1st Generation culture of active control constructed wetland soil (Figure 3.6 A1 and B1), methane concentration increased before dechlorination started, probably because of the abundance of indigenous methanogens existing in the original soils as found in another microcosm study conducted in our lab. In that study, methane concentration was observed to start accumulating immediately after incubation in uncontaminated natural and constructed wetland soils microcosms (data not shown). The observed decrease in methane concentration after dechlorination started was probably due to the removal of methane from the headspace during sampling coupled with ceasing of methane production. Another possible reason is that the total headspace pressure increased due to the production of other gases such as CO_2, resulting in the decrease in the methane partial pressure and consequently its headspace concentration. These indicate that dechlorinators were more competitive for hydrogen than methanogens, although initially methanogens were probably the main microbial group in the microcosms. These observations support the second hypothesis stated above. However, it is also possible that methanogens were responsible for dechlorination since the methanogens, which were used to carbon dioxide

as an electron acceptor, needed some time to get acclimatized to chlorobenzenes as substrates. Once methanogens were adapted to chlorobenzenes, methanogenesis ceased since chlorobenzenes are more attractive electron acceptors than CO_2 by virtue of their high energy yield.

- **BES**

In BES-amended microcosms, aqueous concentrations of methane were all less than 12 μM (μMol/L). However, aqueous concentrations of methane reached a value as high as about 7000 μM (μMol/L) (a mean of triplicates) in active control microcosms during the incubation period. It is worth noting that the solubility of methane in water under 100 psi (6.7 atm) at 25 °C is about 9500 μM (μMol/L) (Carroll et al., 1998), showing that methane concentration and pressure in some of the active control microcosms were substantially high. Lower methane concentration in BES-amended microcosm compared with active control microcosms indicates that methanogenesis was probably partially inhibited by BES as Middeldorp et al. (1997) speculated. Therefore, BES is probably not a very effective complete inhibitor of methanogenesis. In a study conducted by Belay and Daniels (1987), it was found that BES could completely inhibit two *Methanococcu*s species, but it only partly inhibited two *Methanobacterium* strains. In addition, they found that several methanogenic bacteria could use BES to produce ethylene when exposed to the coenzyme M analog (Belay and Daniels, 1987). In the current study, ethylene was observed in some of BES-amended microcosms (data not shown), in agreement with the findings of Belay and Daniels (1987). Therefore, the potency of BES differs depending on the types of methanogenic organisms involved. It is capable of supporting the growth of some methanogens and inhibiting others.

In BES-amended microcosms, the relationships between dechlorination and methane concentration levels were slightly different from that observed in active control microcosms. In some BES-amended microcosms, similar relationship between dechlorination profile and methane concentration pattern as that established in the active control microcosms was found. However, significantly different correlation was observed in the other BES-amended microcosms as discussed below.

During the dechlorination period, methane concentration continued to increase while significant amounts of intermediate daughter products (trichlorobenzenes and dichlorobenzenes) were detected, contrary to the trend observed in the active control microcosms. This phenomenon was observed in the 1st, 2nd and 3rd Generation cultures of BES-amended constructed wetland soil (Figure 3.6 A4 through B6), the 3rd Generation culture of BES-amended natural wetland soil (Appendix I (Figure A6 and B6)) and the 1st Generation culture of BES-amended PPI soil (Appendix III (Figure E6 and F6)). These results indicate that BES could not effectively inhibit methanogens (Belay and Daniels, 1987; Middeldorp et al., 1997; Löffler et al., 1997). In the 3rd Generation cultures of BES-amended constructed wetland soil and natural wetland soil, the parent compound and intermediate daughter products (trichlorobenzenes and dichlorobenzenes) were completely dechlorinated. However, significant amounts of those intermediate daughter products were observed in those BES-amended microcosms, although methane concentration increased continuously during the dechlorination period. These findings, therefore, suggest that dechlorination of chlorobenzenes was probably not mediated by methanogens. Moreover, it is most likely that BES inhibited chlorobenzene dechlorination to a limited extent since trichlorobenzenes and dichlorobenzenes

accumulated. These observations are consistent with the findings by Löffler et al. (1997). In the reported study, it was suggested that dechlorination reactions were catalyzed by bacterial processes rather than methanogenic cometabolism, and that BES had an inhibitory effect on chloroethene dechlorination in the cultures not containing methanogens (Löffler et al., 1997). Middeldorp et al. (1997) also observed complete inhibition of dechlorination of chlorinated benzenes and polychlorinated biphenyls (PCBs) by the addition of BES.

3.3.4 Diversities of Microbial Communities

Soil slurry samples from active control and BES-amended microcosms of all the test soils were collected and analyzed to compare the diversities of microbial communities and to investigate the effects of BES on microbial consortia. Samples from the 3rd Generation cultures of natural wetland soil and river sediment, and the 2nd Generation cultures of constructed wetland soil and PPI soil were taken for DNA extraction and amplification. However, DNA extraction and purification, and PCR amplification of PPI soil slurry sample failed and thus DGGE analysis of microbial community diversity could not be conducted for PPI soil. The failure of DNA extraction and amplification for PPI soil was possibly due to inhibition.

PCR amplification products from two different sets of primers were tested for the presence of bacteria and archaea, and the concentration of amplified PCR products prior to DGGE analysis. Bacteria DNA with the anticipated size of 625 base pair (bp) and archaea DNA with the expected size of 615 bp were detected by the Bioanalyzer for all samples.

- **Bacteria Group**

DGGE band profiles of the PCR amplification products obtained with target DNA of bacteria extracted from active control microcosms of the test soils are shown in Figure 3.7. Comparing the banding patterns (Figure 3.7), the dechlorinating cultures in different test soils contained different microbial composition and diversity. For example, band d existed in constructed wetland soil (Figure 3.7, Lane 1) but probably was not manifested in river sediment (Figure 3.7, Lane 3). In addition, band a was discernable in Lane 1 and Lane 3, but not in Lane 2 (Figure 3.7); whereas band c was visible in Lane 1 and Lane 2 but not in Lane 3 (Figure 3.7). These observations suggest that different microbial communities were able to completely dechlorinate 1,2,3,4-TeCB in active control treatments of all test soils.

Comparison of numbers and distribution patterns of DGGE bands revealed that diversities of the microbial populations in active control and BES-amended microcosms were different for the same type of soil as Figure 3.8 shows. In constructed wetland soil, band A was probably visible in BES-amended treatment but not in active control treatment (Figure 3.8, Lane 1 and 2); whereas band D was intensive in active control treatment but very faint in BES-amended treatment (Figure 3.8, Lane 1 and 2). These differences in the DGGE banding patterns indicate that BES probably altered the microbial compositions. In active control and BES-amended river sediment, bands B, C, E, and F in BES-amended treatment were very faint or probably did not exist in active control treatment (Figure 3.8, Lane 5 and 6), indicating that BES changed the microbial composition and diversity. Chiu and Lee (2001) also observed a DGGE band, which represented a bacterium in the stock culture capable of dechlorinating TCE, whereas the

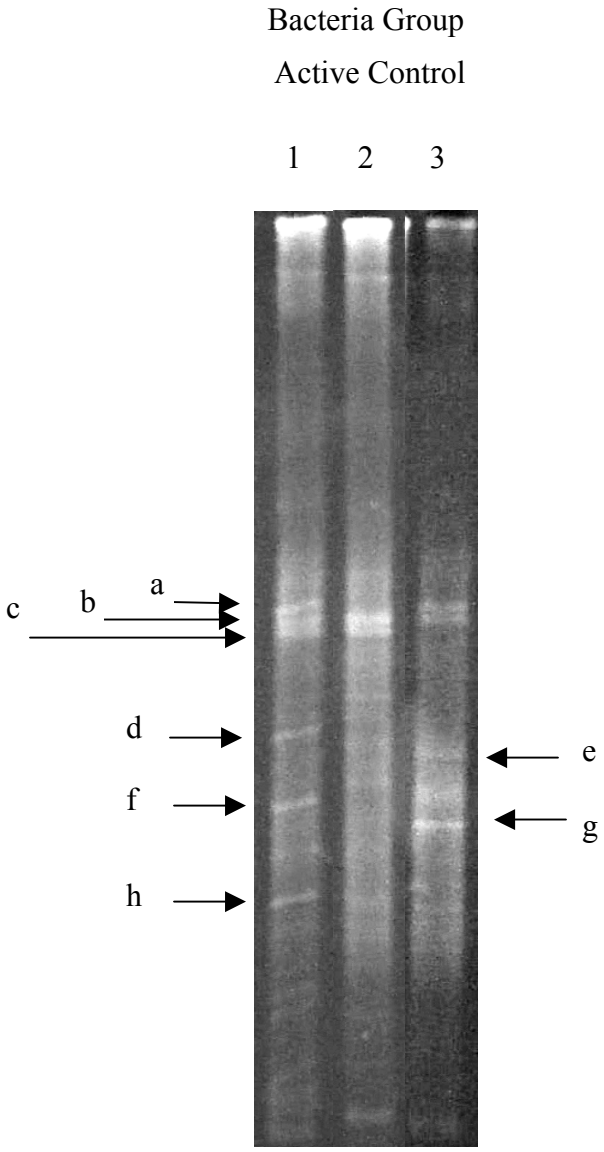

Figure 3.7: DGGE band profile using bacteria primers for active control microcosms of the test soils.

Lane 1: Active control constructed wetland soil;
Lane 2: Active control natural wetland soil;
Lane 3: Active control river sediment.

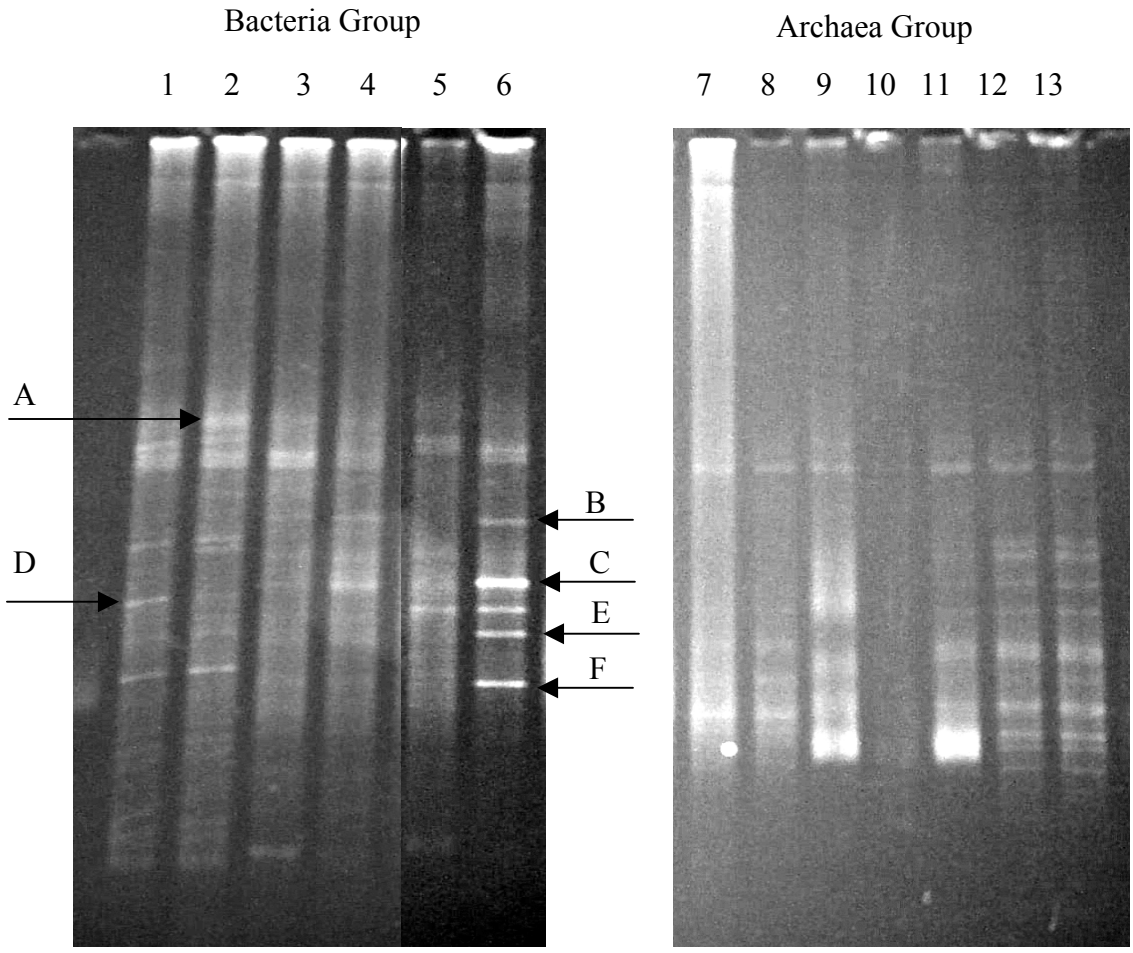

Figure 3.8: DGGE fingerprints of PCR products of bacteria and archaea for comparison of microbial diversities in active control and BES-amended microcosms of the test soils. Lanes 1 to 6 are products from bacteria primers. Lanes 7 to 13 are products from archaea-specific primers.
Lanes 1 and 9: active control constructed wetland soil;
Lanes 2 and 10: BES-amended constructed wetland soil;
Lanes 3 and 11: active control natural wetland soil;
Lanes 4 and 12, 13: BES-amended natural wetland soil (Lane 13 is a replica of Lane 12);
Lanes 5 and 7: active control river sediment;
Lanes 6 and 8: BES-amended river sediment.

same band was not visible in DGGE band profile of BES-amended culture, in which TCE was also biodegraded. On the other hand, a new high intensity band of an uncultured bacterium found in an anaerobic digester appeared in BES-amended culture, however, the same band was very faint in the stock culture (Chiu and Lee, 2001). It is likely that some bacteria could use BES as a substrate, and that BES may also selectively exclude some bacteria, and thus change the microbial structure and diversity. It should be noted that few and faint bands observed in active control river sediment could also have been caused by the insufficient amount of DNA loaded into the DGGE well.

Based on the above results, chlorobenzenes could be biodegraded by different microbial consortia, since chlorobenzene dechlorination occurred in all those cultures. Moreover, comparing DGGE band patterns, some common bands appear in both active control and BES-amended microcosms of all test soils. These bands might represent the dechlorinating bacteria since chlorobenzenes dechlorination occurred in both active control and BES-amended microcosms of all test soils.

- **Archaea Group**

To further investigate the effects of BES on dechlorination and to establish whether methanogens were responsible for dechlorination, DGGE analysis of samples from both active control and BES-amended microcosms was conducted. The band profile for the archaea group is shown in Figure 3.8.

In natural wetland soil, both highly intense and faint bands were observed in active control microcosms; whereas BES-amended microcosms had approximately same number of bands but with nearly the same intensities (Figure 3.8 Lanes 11 and 12, 13). These indicate that BES probably changed the archaea community. Belay and Daniels

(1987) found that BES could completely inhibit two *Methanococcu*s species, but it partly inhibited two *Methanobacterium* strains.

In constructed wetland soil, although intense bands are clearly seen in the active control microcosms, no visible bands are found in BES-amended microcosms (Figure 3.8 Lanes 9 and 10). However, a trace amount of the archaea DNA was detected by the Bioanalyzer in the PCR product from BES-amended microcosm of constructed wetland soil, which resulted in an insufficient amount of DNA for band detection in DGGE. A trace amount of DNA obtained could be due to the inefficient DNA extraction and amplification. However, the detection of DNA amplified using bacteria primers suggests that inefficient DNA extraction may not be the cause. Instead, inefficient DNA amplification or insufficient loading into the gel could be the reason. Otherwise, this result suggests that BES significantly inhibited archaea and thus methanogens in constructed wetland soil. Löffler et al. (1997) also did not detect methanogens by agarose gel electrophoresis in BES treatment after four serial transfers.

Therefore, BES probably changed the archaea community. The inhibitory effects of BES appear to depend on the type of archaea including methanogens and the characteristics of the soils containing these microorganisms.

3.4 Conclusions

1,2,3,4-TeCB was able to be completely biodegraded in all organic matter- and mineral-dominated soils under anaerobic conditions. The dominant pathway of 1,2,3,4-TeCB dechlorination was: 1,2,3,4-TeCB → 1,2,3-TCB → 1,2-DCB + 1,4-DCB + 1,3-DCB → monochlorobenezene + benzene. The results of DGGE analysis showed that different microbial communities with different microbial compositions and diversities

were able to biodegrade 1,2,3,4-TeCB under anaerobic condition. Dechlorination kinetics of chlorobenzenes depends on other factors besides the organic carbon content.

Based on the results of DGGE analysis, it can be observed that BES probably changed the compositions of bacteria and archaea consortia. Moreover, from the results of dechlorination kinetics, hydrogen and methane concentrations, it can be concluded that methanogens were not directly responsible for dechlorination of chlorobenzenes and that BES probably inhibited chlorobenzenes dechlorination to a limited extent and that BES is not an effective complete inhibitor of methanogenesis.

Generally, there was no clear relationship between hydrogen concentrations and methanogenesis or dechlorination. However, hydrogen concentration trends in some treatments suggested that hydrogen was probably used as an electron donor during methanogenesis and for driving dechlorination reactions. Methane started to accumulate after 1,2,3,4-TeCB and its intermediate daughter products (trichlorobenzenes and dichlorobenzenes) were completely degraded in most active control microcosms, indicating that dechlorinators may have out-competed methanogens for electron donors.

Higher dechlorination kinetic constant and shorter lag period can be expected with multiple inoculations, if soil characteristics and experimental conditions remain unchanged. Dechlorinating microorganisms could most likely be adapted to chlorobenzenes. Moreover, complete dechlorination of the parent compound and intermediate daughter products (trichlorobenzenes and dichlorobenzenes) were observed in the 3rd Generation cultures of active control microcosms of all test soils. These observations provide strong evidence for the application of bioremediation (e.g., natural attenuation or *ex-situ* bioremediation). In addition, constructed wetland soil was found to

be a very promising material for *ex-situ* bioremediation of chlorinated solvents contaminated sites using treatment wetland systems. Moreover, natural wetland soil is capable of intrinsic attenuation of chlorinated benzenes.

CHAPTER 4. EFFECTS OF *TYPHA LATIFOLIA* ROOTS ON DECHLORINATION OF 1,2,3,4-TETRACHLOROBENZENE

4.1 Introduction

Uses of plants and associated rhizosphere microorganisms to remove, transform, or contain toxic chemicals is known as phytoremediation (Susarla et al., 2002). Phytoremediation, a relatively new technology, shows great promise as an effective and inexpensive strategy for *in-situ* and *ex-situ* bioremediation (Anderson et al., 1993; Erickson et al., 1994; Cunningham and Ow, 1996; Wiltse et al., 1998; Susarla et al., 2002). Biodegradation of many organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) and TCE has been shown to be enhanced in the presence of plant roots (Anderson and Walton, 1995; Pardue et al., 1996; Nichols et al., 1997; Banks et al., 1999; Siciliano et al., 2003).

A recent study conducted by Siciliano et al. (2003) has revealed that the effectiveness of phytoremediation is plant species dependent. In that study, it was observed that Tall Fescue enhanced the degradation of naphthalene; whereas Rose Clover depressed it (Siciliano et al., 2003). Wiltse et al. (1998) also found variability in degradation of crude oil among genotypes of Alfalfa. Based on these findings, it is, therefore, important to select appropriate types of plants for bioremediation of chlorinated benzenes contaminated sites.

Phytoremediation of contaminated sites involves a number of mechanisms including phytoaccumulation/phytoextraction, phytopumping, phytostabilization and rhizodegradation (Pardue et al., 1996; Susarla et al., 2002; Siciliano et al., 2003). Schnoor et al. (1995) reported that hydrophobic chemicals (Log K_{ow} > 3.0) are bounded to the

surface of roots so strongly that they can not be translocated within the plant. Since chlorobenzenes (i.e., tetrachlorobenzenes, trichlorobenzenes and dichlorobenzenes) have low water solubilities (Log K_{ow} > 3.0) (EPA, 1966; Site, 2001), plant uptake of chlorinated benzenes from contaminated soils through phytopumping or phytoaccumulation/phytoextraction is limited. In addition, there is little data available on plant-induced sequestration (phytostabilization) of organic contaminants in soils (Siciliano et al., 2003). Therefore, the most important mechanism involved for enhancement of chlorobenzenes biodegradation is probably rhizodegradation, a biological treatment by enhanced microbial activity in the rhizosphere.

The rhizosphere, the area around plant roots, has generally greater microbial activities as a result of energy and carbon sources provided by rhizodeposition (Pardue et al., 1996; Haby and Crowley, 1996). A significant part of organic matter released from living roots comprises water-insoluble materials such as freed cap cells and lysates, and water-soluble exudates such as organic acids and sugars, and other root secretions such as enzymes (Whipps, 1990; Anderson et al., 1993; Brimecombe, et al., 2001). These materials can not only serve as substrates to nearby microorganisms, but they will also induce changes in the physicochemical characteristics of the surrounding soil, and thus, change the microbial diversity (Marilley et al., 1998; Brimecombe, et al., 2001). The rhizosphere has been reported to contain higher populations and greater diversities of microbial consortia (Anderson et al., 1993; Nichols et al., 1997; Siciliano et al., 2003), which may increase biodegradation activities. Moreover, some organic acids, as components of root exudates, could serve as electron donors for dechlorination (Holliger

et al., 1992; Middeldorp et al., 1997), which may also contribute to the enhanced biodegradation in the rhizosphere.

Few, if any study has been undertaken on the effects of wetland plants on biodegradation of chlorinated benzenes. *Typha latifolia L*. is a native wetland plant that can grow prolifically from thick underground rhizomes. Therefore, the present study was conducted to investigate the effects of *Typha* roots on dechlorination of 1,2,3,4-TeCB, organic acids production and microbial population diversities in soil. Anaerobic microcosm studies on dechlorination of 1,2,3,4-TeCB in river sediment amended with different amounts of *Typha* roots were conducted. The objectives of the study were (i) to determine the effects of *Typha* roots on dechlorination kinetics and pathways; (ii) to investigate the effects of *Typha* roots on the diversities of microbial consortia in soil; and (iii) to correlate the amount of roots with hydrogen, methane and organic acids concentrations. The results of the present study may be useful in assessing the feasibility of using *Typha* for *in-situ* and *ex-situ* bioremediation of sites contaminated with chlorobenzenes.

4.2 Materials and Methods

4.2.1 Chemicals

Neat 1,2,3,4-TeCB from Supelco (Bellefonte, PA) was used as the test chemical in this study. 1,2,3-TCB, 1,2,4-TCB, 1,3,5-TCB, 1,2-DCB, 1,3-DCB and 1,4-DCB used for calibration were analytical grade from Sigma-Aldrich. Standards for benzene and monochlorobenzene, internal standards and surrogates for EPA Method 8260 were procured from Supelco. Methane used for calibration was also obtained from Supelco

Inc. (Supelco, Bellefonte, PA). Hydrogen standards were obtained from BOC Group Inc. (Baton Rouge, LA). HPLC grade hexane and methanol were used as solvents.

4.2.2 Soil Collection and Root Preparation

River sediment was obtained from Bayou Duplantier, Baton Rouge, Louisiana. Live *Typha* plants were collected from a freshwater wetland in Madisonville, Louisiana, and then transplanted in greenhouse for about one month. Live roots of *Typha* were washed several times with tap water followed by deionized water. Fresh roots were finally cut by sterile razor and immediately used for preparing microcosms.

4.2.3 Microcosm Experiment

Anaerobic microcosms were set up in a glove bag (I^2R, Cheltenham, PA) under nitrogen atmosphere. Homogenized river sediment and different amounts of roots were packed in 160 mL serum bottles leaving 80 mL headspace. River sediment was mixed with 1, 2 and 5g of roots for a total of 43 g in each serum bottle. One set of microcosm was prepared without roots as a control. Therefore, the experiment involved the following four treatments as tabulated in Table 4.1. All treatments were prepared in triplicate.

Table 4.1: List of treatments based on the amounts of *Typha* roots.

Treatments	Mass of roots, g	Mass of sediment, g	Ratio of roots to sediment, R/S (g/g), %
No roots (RCNR)	0	43	0
Small amount of roots (RCSR)	1	42	2.4
Medium amount of roots (RCMR)	2	41	4.9
Large amount of roots (RCLR)	5	38	13.2

A volumetric ratio of water to sediment of 1.5: 1 was used (Lorah et al., 1997). Microcosms were prepared using deionized water. All bottles were sealed with Teflon-

lined rubber septa and aluminum crimp seals and incubated under static and dark conditions at 25 °C. Microcosms were neither amended with electron donors nor nutritional supplements to support microbial growth.

1,2,3,4-TeCB was dissolved in methanol (Pavlostathis and Prytula, 2000) and then spiked into microcosms to a final concentration of about 150 mg/kg dry weight of soil. A relatively small volume of methanol of between 0.5 mL and 1 mL (in a total of 140 mL slurry) was used for spiking the test chemical in order to limit the effects of methanol on dechlorination. To minimize the amount of methanol, the bottles were purged with nitrogen at 1 atm using a syringe needle for about 1 min and immediately sealed inside the glove bag. Two identical sets of microcosms were set up for each treatment. One set was used for gas analysis and the other set was used for chlorobenzenes analysis and molecular analysis.

Concentrations of the parent compound and degradation daughter products were monitored until the concentration of the parent compound had dropped below the detection limit of the analytical methods (5 ng/μL in the hexane extract). Slurry sampling for analysis of chlorobenzenes was done inside the glove bag (I^2R, Cheltenham, PA) in order to maintain anaerobic conditions in the microcosms. Four mL of soil slurry was withdrawn from microcosms after shaking the bottle to homogenize the contents, the bottle was flushed with nitrogen at 1 atm for about 1 min and resealed. The soil slurry was then transferred into Teflon centrifuge tube to minimize the adsorption of chlorinated benzenes. An equal volume of hexane (i.e., 4 mL) was immediately added into the Teflon centrifuge tubes (Holliger et al., 1992; Chang et al., 1997; Chen et al., 2002). The mixture of slurry and hexane was then tumbled for 24 hours to facilitate the extraction of

chlorobenzenes. The suspension was centrifuged at 3,000 rpm for about 15 minutes at room temperature and 1 mL of supernatant was transferred into an amber GC-MS vial for analysis of semivolatile chlorobenzenes (i.e., tetrachlorobenzenes, trichlorobenzenes and dichlorobenzenes).

Aqueous samples for analysis of benzene and chlorobenzenes were directly withdrawn from microcosms using a gas tight syringe and transferred into autosampler vials. Aqueous samples for analysis of organic acids were withdrawn from the serum bottles using a sterile syringe, filtered through a 0.2 μm syringe filter, and finally preserved with 8 N phosphoric acid (5 μL per 1 mL sample) in autosampler vials (Pardue, et al., 2001). Gas samples for analysis of methane and hydrogen were analyzed without storage.

4.2.4 Analytical Procedures

The hexane extract was analyzed following EPA Method 8270 for the measurement of semivolatile chlorinated benzenes (i.e., tetrachlorobenzenes, trichlorobenzenes and dichlorobenzenes). Ten μL of semivolatile internal standards mix (2000 μg/mL in methylene chloride, containing 1,4-dichlorobenzene-d_4, naphthalene-d_8, acenaphthalene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}) (Supelco Chemical Co.) was injected into 1 mL hexane extract. The sample was then analyzed by GC-MS (Agilent 6890 series gas chromatograph-5972A mass selective detector). The GC was equipped with a capillary column (DB-5, 30 m × 0.25 mm i.d., 0.25 μm film thickness) which was directly interfaced to the mass spectrometer. High pure helium was used as a carrier gas at a flow rate of 1.8 mL/min. The injector temperature was 250 °C. The GC column was initially held at 37 °C for 2 min, then ramped to 260 °C at 8 °C/min, and

finally ramped to 300 °C at 40 °C /min and held for 10 min. The detector temperature was maintained at 280 °C.

Analysis of benzene and chlorobenzene were performed by EPA Method 8260B using a purge and trap apparatus attached to a Agilent 6890 Series Gas Chromatograph equipped with a 5972A mass selective detector. A thermal desorption trap (VOCARB 3000; Supelco, Bellefonte, PA) was employed in the purge and trap apparatus. The hexane extract along with 10 μL internal standard and 2.5 μL surrogate (Supelco, Bellefonte, PA) was manually injected into the purge and trap autosampler (Tekmar 2016) (Tekmar Dohrmann, Mason, OH), and purged for 11 min with ultra-high-pure helium at a flow rate of 35 mL/min, then desorbed for 0.5 min and baked for 13 min at 225 °C. The samples were then introduced onto the GC equipped with a 60 m × 0.32 mm × 3.00 μm film thickness, Agilent 5MS (Palo Alto, CA) capillary column (Palo Alto, CA). High purity helium gas was used as a carrier at a flow rate of 2.1 mL/min. The GC column temperature program was 35 °C for 5 min, and ramped at 4 °C/min to a final temperature of 200 °C. The temperatures of injector and detector were 250 °C and 280 °C, respectively.

Prior to sample analysis, six-point calibration curves were established for both methods to determine the relative response factors for the individual compound. Tune, daily blank and calibration check were conducted to assure that the machine and the analytical methods were in control.

Organic acids were analyzed by High Performance Liquid Chromatograph (HPLC) (Dionex LC-20, Dionex Corp., Sunnyvale, CA). Methane was measured by GC-FID. One mL of gas was withdrawn from the headspace of the bottle using a gas tight

syringe, and then injected into GC-FID (Agilent 5890 series II) equipped with a 2.4 m × 0.32 mm i. d. column packed with Carbopack b/l % SP-1000 (Supelco, Bellefonte, PA). The injector and detector temperatures were 375 °C and 325 °C, respectively. The column temperature was held constant at 50 °C for 6.50 min. Ultra high pure nitrogen (BOC Gases, Baton Rouge, LA) was used as a carrier gas at a flow rate of 12 mL/min. All methane data are reported as aqueous concentrations in μM (μmol/L). Headspace methane concentrations were converted to aqueous phase concentrations using Henry's Law (Henry's constant for methane at 25 °C is 0.6364 atm/mol/m^3).

Hydrogen was analyzed using a reduction gas analyzer (Trace Analytical, Menlo Park, CA) equipped with a reduction gas detector. Gas samples taken from the headspace were manually injected into a 1-mL gas sampling loop, and then separated with a molecular sieve analytical column (Trace Analytical, Menlo Park, CA) at an oven temperature of 40 °C. Ultra high pure nitrogen (BOC Gases, Baton Rouge, LA) was used as a carrier gas. The detection limit under these conditions was 1 ppb. All hydrogen data are reported as aqueous concentration.

Aqueous concentration of H_2 was calculated following the equation adopted from Löffler et al. (1999):

$$[H_{2,aq.}] = LP / RT$$

where $H_{2,aq.}$ is the aqueous concentration of H_2 (moles/L);

L is the Ostwald coefficient for H_2 solubility (0.01913 at 25 °C);

P is the partial pressure of H_2 (atm);

R is the universal gas constant (0.0821 liter·atm·K^{-1}·mol^{-1});

and T is the temperature (K).

$$P = C/10^6$$

where C is the gas phase concentration of H_2 (ppm);

4.2.5 Molecular Analysis

- **DNA Extraction**

At the end of incubation period, slurry samples were taken from RCLR and RCNR microcosms after shaking the bottles for homogenization, and then immediately stored in sterile cryogenic vials at -20 °C prior to DNA extraction. An appropriate amount of slurry was extracted following the protocol of Mo Bio Ultraclean Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Salona Beach, CA) with some modifications. Because of the large amount of humic acids in the soil samples which can inhibit PCR amplification, samples were treated with polyvinylpolypyrrolidone (PVPP) (Agros Organics, Geel, Belgium) (about 0.1 g per 1 g of sample) as a humic acid-binding agent prior to extraction (Holben, et al., 1988). In order to further remove traces of humic acids, two additional washes using S4 solution (a component of Ultraclean Soil DNA Isolation Kit) were performed. Another modification of the kit protocol was that a Biospec Mini-Beadbeater 3110BX (Biospec products Inc., Bartlesville, OK) was utilized for cell disruption instead of Mo Bio Vortex Adapter (Mo Bio Laboratories, Inc.). The beadbeater was operated at 4,800 rpm for 3.0 min. Extracted DNA was stored at –20 °C until further analysis.

- **PCR Amplification**

Extracted DNA was amplified by Polymerase Chain Reaction (PCR) using an Eppendorf MasterTaq Kit (Brinkmann Instruments, Inc., Westbury, NY). The Eppendorf MasterTaq Kit includes Taq DNA Polymerase (5 U/ μL), 10 × Taq Buffer with Mg^{2+}, and

5 × TaqMaster PCR Enhancer. The 5 × TaqMaster PCR Enhancer often required heating at 60 °C to dissolve the components completely. The master mix was made of 63.5 μL 18 Mega Ohm water, 15 μL of 5 × TaqMaster PCR Enhancer, 10 μL of 10 × Taq Buffer with Mg^{+}, 8 μL of the 10 mM dNTP mix (Applied Biosystems, Forster City, CA), 0.5 μL of the Taq DNA polymerase and 1 μL of each primer (forward and reverse) per sample. For each sample to be amplified, 99 μL of the master mix was placed in a 500-μL sterile PCR reaction tube, and then 1 μL of the extracted DNA was added. This mixture was vortexed and then centrifuged for 1 min at 13,000 rpm. PCR amplification was finally performed by an Eppendorf Thermocycler (Eppendorf GmbH, Hamburg, Germany).

Two different types of primers were applied. One was 341f (5'-CCTACGGGAGGCAGCAG-3') and 907r (5'-CCGTCAATTCMTTTRAGTTT-3') (Casamayor et al., 2000) for the bacteria group; the other set of primers for the archaea group (i.e., methanogens) was archaeon-specific primers 340f (5'-CCTACGGGGCGCASCAGGSGC-3') and 915r (5'-GTGCTCCCCCGCCAATTCCT-3') (Löffler et al., 1997). An additional 40-nucleotide GC-rich sequence (GC-clamp) attached to the 5' end of both forward primers was: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG (Muyzer et al., 1995). All these primers were obtained from Alpha DNA (Quebec, CA). For the bacteria group, PCR conditions were (Hendrickson et al., 2002): denaturation, 95 °C (2 min); 40 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (1 min) and finally cooling at 4 °C. For the archaea group, the PCR conditions (Löffler et al., 1997) were: denaturation, 94 °C (2 min 10 s); 30 cycles of 94 °C (30 s), 60 °C (45 s), 72 °C (2 min 10 s); final elongation, 72 °C (6 min). PCR products were immediately analyzed or stored at 0 – 4 °C until analysis.

- **Detection of PCR Products**

PCR products were analyzed by the Agilent 2100 Bioanalyzer and corresponding DNA Labchip Kits (Agilent Technology, Willington, DE) to obtain the concentration of DNA and to determine whether the DNA extraction and PCR amplification were successful. One μL of PCR product was used for analysis following the manufacturer's instruction.

- **Denaturing Gradient Gel Electrophoresis (DGGE)**

DGGE was performed using a D-Code^{TM} Universal Mutation Detection System (Bio-Rad, Hercules, CA) as described by Myers et al. (1987) with the following modifications. The 24 mL denaturing gradient gel (6% (wt/vol) acrylamide solution) was covered by a 5 mL acrylamide stacking gel without denaturant. Polymerization was catalyzed with addition of 0.0381% of TEMED (vol/vol) and 0.914% of the 10% ammonium persulfate (vol/vol) to both denaturant solutions. 0.85% of the 10% ammonium persulfate (vol/vol) and 0.057% of TEMED was added to the 0% stacking gel solution. Gels were cast using a BioRad Model 475 Gradient Delivery System. Samples containing approximately equal amounts of PCR amplicons (with loading dye) were loaded into individual gel lanes. The polyacrylamide gels were made with a denaturing gradient ranging from 30% to 80% and from 40% to 70% for bacteria group and archaea group, respectively (100% denaturant contained 42% (wt/vol) urea and 40% (vol/vol) formamide (Bio-Rad, Hercules, CA)). Electrophoresis was performed in 1× TAE buffer at 60 °C for 15 hours at 65 V. Following electrophoresis, the gel was stained with ethidium bromide for 10 min. The gel was then destained using 1x TAE buffer for 12

min. Finally, the gel was visualized with a UV transilluminator, photographed and digitized using an Alpha DigiDoc system (Alpha Innotech Co., San Leandro, CA).

4.2.6 Data Analysis

- **Kinetic Data Modeling**

First-order reaction rate constant was calculated from the first-order kinetic equation as shown below by optimization of degradation kinetic data using non-linear regression techniques. When the amount of daughter products detected at the *i*th sampling point was at least 5% of the parent compound after adjustment at the (*i*-1)th sampling point, the onset of dechlorination was assumed and the lag period was considered to be the time between the *i*th and the (*i*-1)th sampling points.

$$C_t = C_o e^{-kt}$$

where t is the time (day);

C_t is the concentration at any time t (mM/kg dry soil);

C_o is the initial concentration (mM/kg dry soil);

and k is the pseudo first-order reaction rate constant (day^{-1}).

The characteristic half-life period ($t_{1/2}$) was calculated from the first-order reaction rate constant (k) using the following equation:

$$t_{1/2} = -\frac{(\ln 2)}{k} = \frac{0.693}{k}$$

where $t_{1/2}$ is the half-life time (days);

k is the pseudo first-order reaction rate constant (day^{-1}).

- **Statistical Analysis**

First order kinetic rate constants and associated standard errors were calculated from non-linear regression of kinetic data using SigmaPlot 2001 Version 7.0 (SPSS Inc., San Rafael, CA). A two-sample t-test was used to compare the differences in first-order kinetic values between different treatments using a significance level of 5%.

4.3 Results and Discussion

4.3.1 Dechlorination Pathways and Kinetics

- **Dechlorination Pathways**

1,2,3,4-TeCB was completely biodegraded in all treatments. Generally, 1,2,3,4-TeCB was dechlorinated to 1,2,3-TCB, and 1,2-DCB and 1,4-DCB, and finally to chlorobenzene and/or benzene. Significant amounts of chlorobenzene and a trace amount of benzene were detected in aqueous samples of all treatments. 1,2,4-TCB was detected in the RCSR treatment only. Dechlorination daughter products in each treatment are listed in Table 4.2. The dechlorination profiles of all treatments are shown in Figure 4.1, 4.3, 4.5 and 4.7. Generally, there were no significant differences in dechlorination products between treatments with and without roots, indicating that probably the same types of microorganisms were involved in dechlorination in all treatments (Table 4.2).

The most dominant dechlorination pathway observed in the present study is very similar to that found by Nowak et al. (1996). In that study, 1,2,3,4-TeCB was dechlorinated to 1,2,3-TCB and all isomers of dichlorobenzenes in 1,3,5-TCB adapted methanogenic consortia. However, the observed dechlorination pathway is different from those reported by Masunaga et al. (1996) and Pavlostathis and Prytula (2000). In those studies, 1,2,3,4-TeCB was mainly degraded to 1,2,4-TCB and small amounts of 1,2,3-

TCB, and all isomers of dichlorobenzenes. Differences in dechlorination patterns were probably due to differences in the microbial communities in which dechlorination occurred in the studies.

Table 4.2: List of daughter products in all treatments.

Treatment	Daughter products	Major intermediate daughter products
Large amount of roots (RCLR)	1,2,3-TCB, 1,2-DCB, 1,4-DCB, chlorobenzene and benzene	1,2,3-TCB, 1,2-DCB, and 1,4-DCB
Medium amount of roots (RCMR)	1,2,3-TCB, 1,2-DCB, 1,4-DCB, chlorobenzene and benzene	1,2,3-TCB, 1,2-DCB, and 1,4-DCB
Small amount of roots (RCSR)	1,2,3-TCB, 1,2,4-TCB, 1,2-DCB, 1,4-DCB, chlorobenzene and benzene	1,2,3-TCB, 1,2,4-TCB, 1,2-DCB, and 1,4-DCB
No root (RCNR)	1,2,3-TCB, 1,2-DCB, 1,4-DCB, chlorobenzene and benzene	1,2,3-TCB, 1,2-DCB, and 1,4-DCB

Intermediate daughter products (trichlorobenzenes and dichlorobenzenes) were not accumulated in RCLR and RCMR treatments (Figure 4.1 and Figure 4.3), whereas substantial accumulations of 1,4-DCB in RCSR treatment (Figure 4.5), and 1,4-DCB and 1,2-DCB in RCNR treatment (Figure 4.7) were observed. These results suggest that the presence of *Typha* roots greatly enhanced the extent of 1,2,3,4-TeCB biodegradation.

- **Dechlorination Kinetics**

Dechlorination rates and lag periods are shown in Table 4.3. Lag periods decreased with increasing amount of roots, for example, lag period in RCLR was 4 – 7 days, whereas lag period in RCNR was 20 - 27 days. 1,2,3,4-TeCB degradation rate

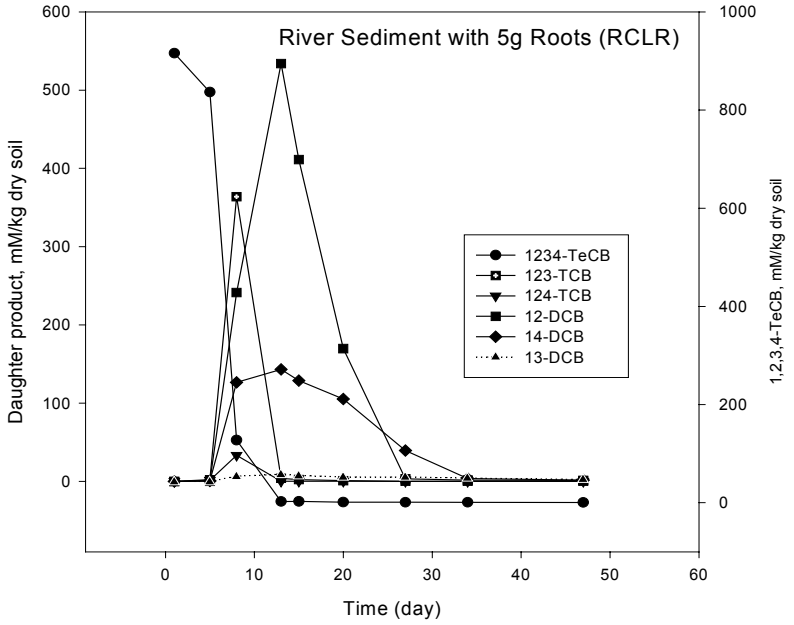

Figure 4.1: Dechlorination profile of 1,2,3,4-TeCB in RCLR treatment. Each data point is the mean of three replicates.

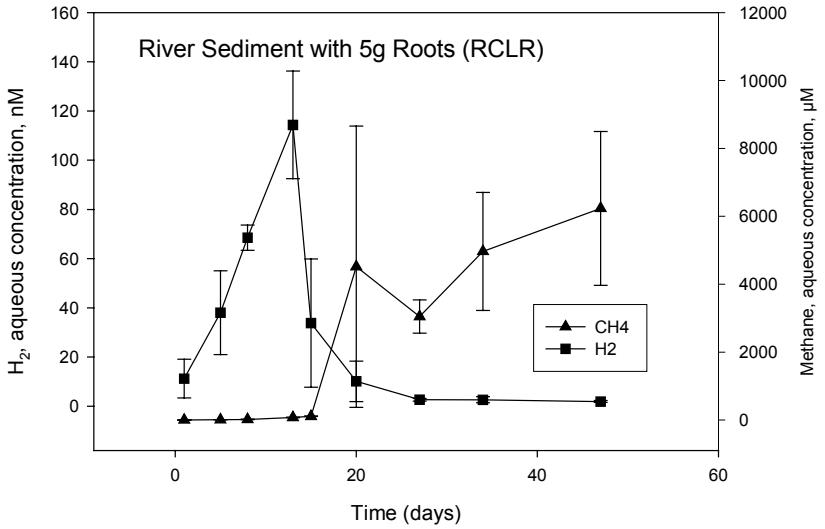

Figure 4.2: Methane and hydrogen concentrations in RCLR treatment.

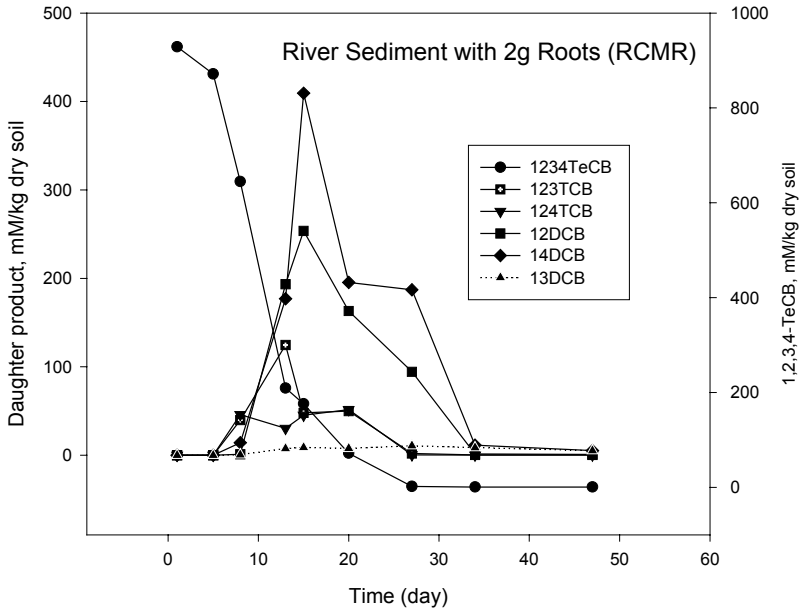

Figure 4.3: Dechlorination profile of 1,2,3,4-TeCB in RCMR treatment. Each data point is the mean of three replicates.

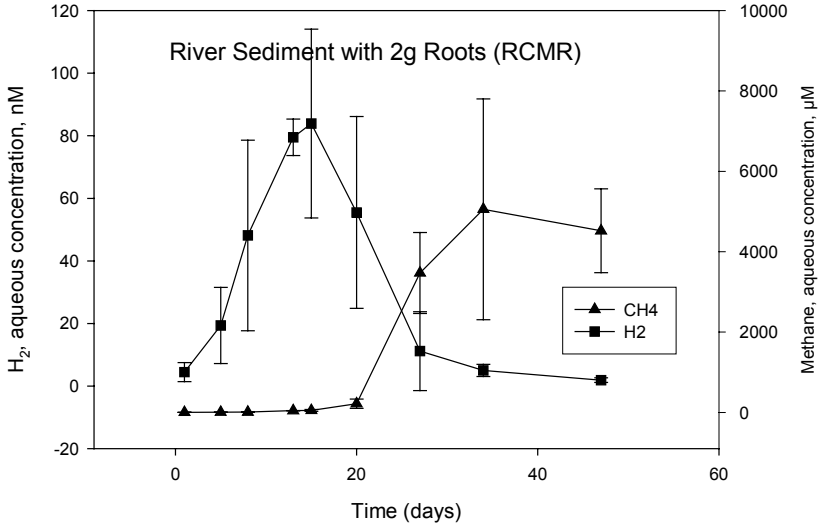

Figure 4.4: Methane and hydrogen concentrations in RCMR treatment.

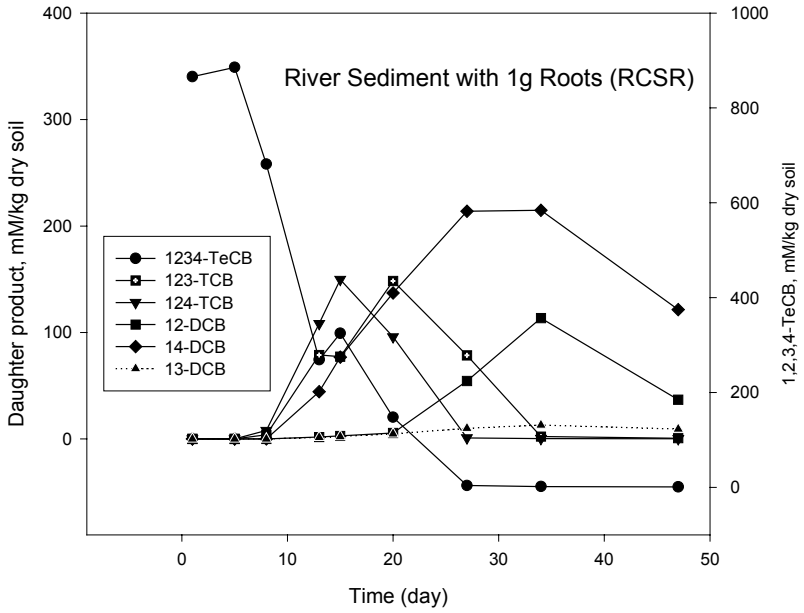

Figure 4.5: Dechlorination profile of 1,2,3,4-TeCB in RCSR treatment. Each data point is the mean of three replicates.

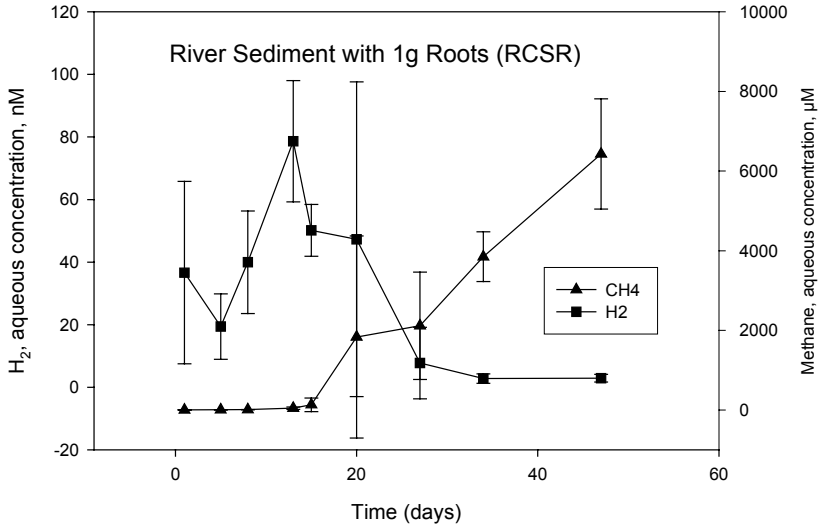

Figure 4.6: Methane and hydrogen concentrations in RCSR treatment.

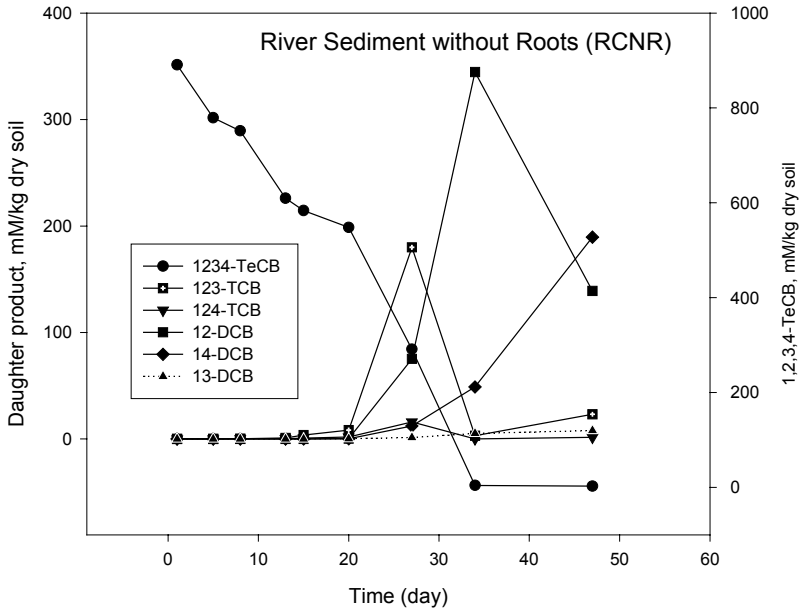

Figure 4.7: Dechlorination profile of 1,2,3,4-TeCB in RCNR treatment. Each data point is the mean of three replicates.

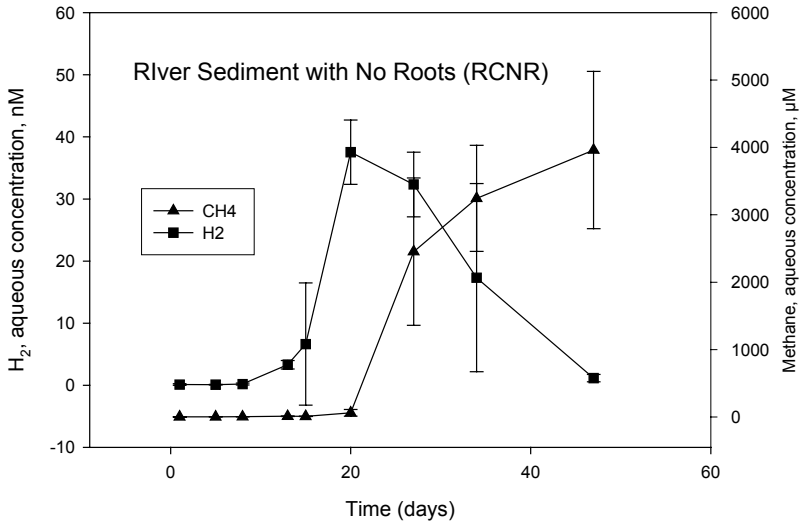

Figure 4.8: Methane and hydrogen concentrations in RCNR treatment.

constants were observed to increase with increasing amounts of *Typha* roots as illustrated in Table 4.3 and Figure 4.9. The first-order dechlorination rate constant in RCLR treatment was about 5 times that observed in the RCNR treatment. The degradation rate constant of 1,2,3,4-TeCB in the RCLR treatment was significantly higher than in all the other treatments (RCMR, RCSR, RCNR, and RBMR) ($P > 0.05$) (Table 4.3). These results indicate that the root matter of *Typha* has the potential to enhance the biodegradation rate of 1,2,3,4-TeCB, probably because *Typha* roots benefited the dechlorinating microorganisms. Similarly, Jordahl et al. (1997) observed higher populations of benzene-, toluene-, and o-xylene-degrading bacteria in the rhizosphere of poplar trees than in the non-rhizosphere soil. Biodegradation of many organic contaminants such as TCE, 3-chlorobenzoate, benzo[a]pyrene and crude oil was reported to be promoted in the rhizosphere compared to non-vegetated soil (Anderson and Walton, 1995; Haby and Crowley, 1996; Wiltse et al., 1998; Banks et al., 1999). The findings of the current study are, therefore, in agreement with the studies reported above.

Shorter lag times and higher kinetic rates in the treatments with roots, compared with the treatment without roots, were probably caused by the carbonaceous root matter, which provided appropriate conditions and substrates for microorganisms to grow (Gilbert et al., 1996; Susarla et al., 2002). For example, volatile fatty acids from roots may be direct electron donors or precursors of hydrogen required for driving degradation reactions of organic contaminants (Holliger et al., 1992; Middeldorp et al., 1997).

However, roots of some plants may inhibit degradation of contaminants. In a recent study, Siciliano et al. (2003) found that mineralization of phenanthrene decreased in the rhizosphere of Rose Clover. Differences in the effects of rhizosphere on

Table 4.3: Dechlorination kinetic rate constants, half-life times and lag periods.

Treatments	Lag period, days	K, day^{-1}	$t_{1/2}$, days	R^2	95% confidence interval of K
River sediment with large amount of roots (RCLR)	4-7	0.63±0.004	1.1	0.99	0.62 – 0.64
River sediment with medium amount of roots (RCMR)	4-7	0.16±0.01	4.4	0.99	0.13 – 0.19
River sediment with small amount of roots (RCSR)	8-13	0.14±0.02	5.0	0.97	0.10 –0.18
River sediment with no roots (RCNR)	20-27	0.13±0.04	5.3	0.94	0.05 – 0.21

Note: K: pseudo first-order kinetic constant, day^{-1}; ±: standard error of the pseudo first-order kinetic constant from the non-linear regression; $t_{1/2:}$ half-life time, days; R^2: coefficient of determination for the non-linear regression.

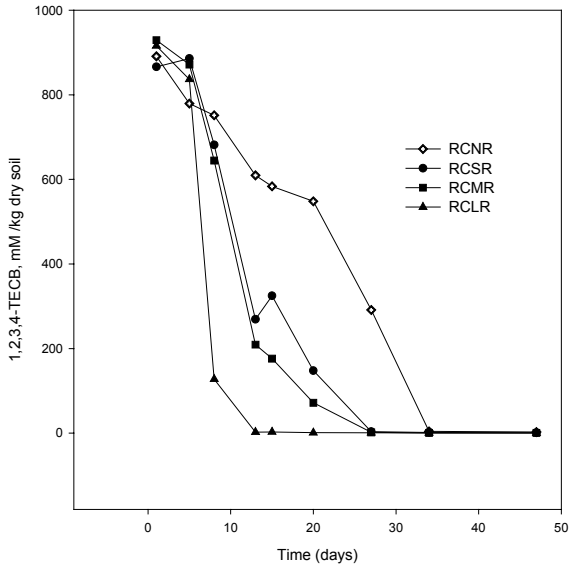

Figure 4.9: Dechlorination of 1,2,3,4-TeCB in treatments with different amounts of roots.

biodegradation of organic pollutants are speculated to be due to the differences in the interaction of plant roots exudates and microorganisms, probably caused by the differences in the characteristics of plant roots such as alterations in root exudate patterns and root architecture (Marilley et al., 1998; Siciliano and Germida, 1999; Siciliano et al., 2003). Cieslinski et al. (1997) found that exudation of low molecular mass organic acids in the rhizosphere of wheat and flax differed significantly between cultivars. Soil acidity, redox potential, oxygen availability and other parameters in the rhizosphere may be altered by the root exudates, which in turn may influence the microbial diversity in the soil and thus affect rhizodegradation (Marilley et al., 1998). Moreover, varied performances of rhizodegradation may also be caused by differences in root morphology such as root density and abundance (Siciliano et al., 2003). Based on the above discussion, selection of the appropriate type of plant for application in bioremediation is very important. Since dechlorination of the test chemical was enhanced by *Typha*, the plant could be a very promising vegetation for phytoremediation of chlorobenzene contaminated sites.

4.3.2 Organic Acids, Hydrogen and Methane Concentrations

Generally, acetic and propionic acids were the most abundant organic acids detected. Concentrations of these organic acids were observed to increase with increasing amount of *Typha* roots as Figure 4.10 and 4.11 illustrate. This observation indicates that *Typha* root matter increased the production of organic acids. Higher concentrations of organic acids in root-amended microcosms compared to non-amended soil could be due to fermentation of root organic matter and root exudates. Conrad and Klose (2000) also

reported that a number of fatty acids including acetate, propionate and butyrate were produced by washed excised roots of rice (Oryza sativa).

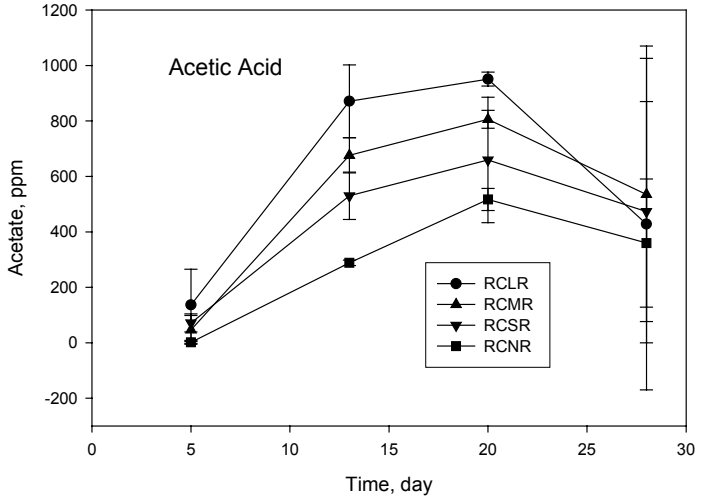

Figure 4.10: Acetic acid concentrations in all treatments.

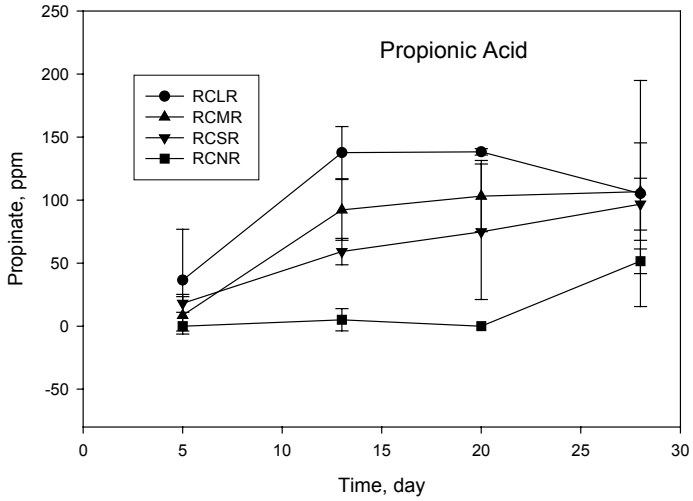

Figure 4.11: Propionic acid concentrations in all treatments.

Concentration of acetic acid was significantly higher than those of other organic acids detected including propionic, butyric, lactic, benzoic, and formic acids (data not

shown), probably because of the fermentation reactions by acetogens (Fennell and Gossett 1997; Fang and Jia, 1999). Other fatty acids were converted to acetate and CO_2/H_2 through fermentation as Table 4.4 shows. This may also explain the observed increase in H_2 concentration with increasing amount of *Typha* roots (Figure 4.2, 4.4, 4.6, and 4.8), considering that acetate concentration also increased with increasing amount of roots (Figure 4.10). High dechlorination activity in treatments with roots probably was due to the abundance of hydrogen and organic acids such acetate and propionate, which were able to serve as electron donors for dechlorination (Holliger et al., 1992; Middeldorp et al., 1997). In addition, higher concentration of propionic acid was observed in root-amended microcosm than in unamended soil. Since propionic acid degrades slowly and provides a slow and steady release of low levels of H_2, thus dechlorination may be favored over competing methanogenesis (Fennell and Gossett, 1997). Fennell and Gossett (1997) observed accumulation of propionic acid in their PCE dechlorinating culture, which facilitated continued dechlorination after the primary donors were depleted.

Table 4.4: Fermentation reactions of fatty acids.

Fermentation of fatty acids to acetate and H_2
$Butyrate^- + 2\ H_2O \rightarrow 2\ Acetate^- + H^+ + 2H_2$
$Lactate^- + 2\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2H_2$
$Propionate^- + 3\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$

Faster and transient accumulations of hydrogen (up to 2 weeks after incubation) in root-amended microcosms (Figure 4.2, 4.4, 4.6, and 4.8) were probably caused by fermentations of organic acids produced from *Typha* roots (Table 4.4). Low hydrogen

(Figure 4.8) and organic acids concentrations (Figure 4.10 and 4.11) in RCNR treatment in the first 2 weeks of the incubation compared with root-amended treatments (Figure 4.2, 4.4, and 4.6) may explain the significantly lower dechlorination activity in the absence of roots (Figure 4.7) (i.e., 1,2,3,4-TeCB dechlorination delayed for about 2 weeks in RCNR treatment). Decreases in hydrogen concentration after 2 or 3 weeks of incubation were probably because consumption of hydrogen was much higher than its production. Since hydrogen can be used as an electron donor for both dechlorination and methanogenesis, and production of hydrogen probably decreased due to the decrease in secretion of root exudates with time considering that the roots were not as fresh as they were at the beginning, therefore, hydrogen concentration started to drop. Accumulation of methane coincided with decrease in hydrogen concentration (Figure 4.2, 4.4, and 4.6), supporting the above reasons.

Moreover, faster methane accumulation was observed in root-amended microcosms than in microcosm with soil alone, probably due to the initial faster accumulations of hydrogen and acetate in root-amended microcosms, since both hydrogen and acetate could be converted to methane by methanogens (Fang and Jia, 1999). Fennell and Gossett (1997) also observed a simultaneous increase in methane production and decrease in acetic acid concentration, which was due to significant acetotrophic activity.

4.3.3 Diversities of Microbial Communities

Soil slurry samples from one of the triplicate microcosms of RCLR and RCNR were collected and analyzed using DGGE technique. The DGGE banding profiles are shown in Figure 4.12.

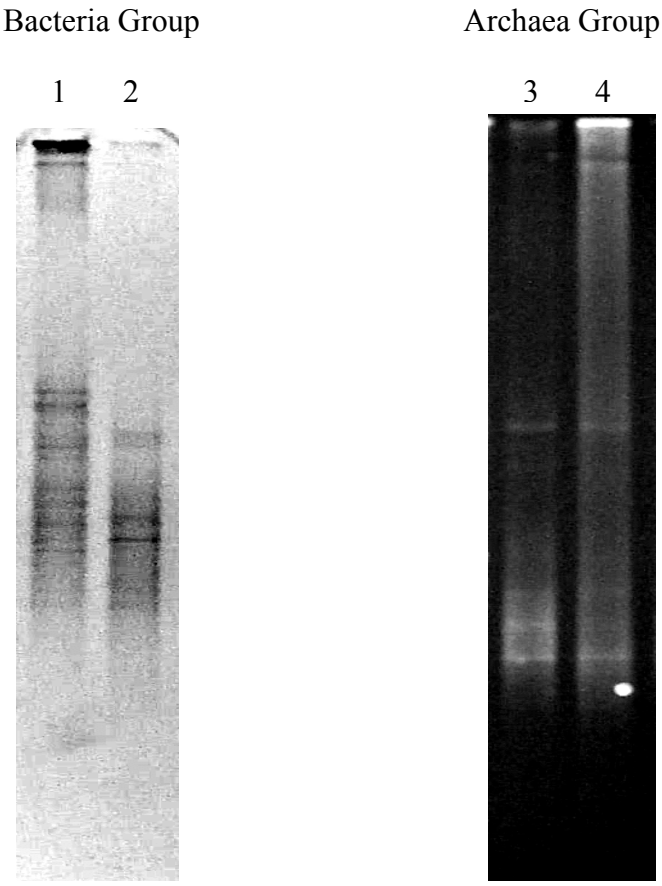

Figure 4.12: DGGE fingerprints for investigation of the effects of *Typha* roots on microbial diversity.
Lane 1 and 3: river sediment with large amount of roots (5g roots) (RCLR).
Lane 2 and 4: river sediment with no roots (RCNR).

Comparing the banding profile of bacteria group (Figure 4.12 Lane 1 and 2), different banding patterns were observed in root-amended and unamended microcosms, indicating that the presence of roots changed the bacterial community. Previous studies have shown that the rhizosphere could increase or decrease the bacterial diversity. Most of the studies have reported greater bacterial diversity in the rhizosphere than in the bulk soil (Campbell and Greaves, 1990; Anderson et al., 1993; Gilbert et al., 1996; Nichols et al., 1997). However, Marilley et al. (1998) observed higher bacterial diversity in the bulk

soil than in the rhizosphere. Siciliano and Germida (1999) also observed cultivar-dependent differences in the root interior microbial community. The differences in these findings were possibly caused by the differences in interactions between root exudates and microorganisms, indicating that the type of plant may derive different alteration in the microbial consortia. The change in the composition and diversity of the bacterial community in the rhizosphere is, therefore, strongly influenced by the characteristics of the plant root such as root exudates and root density, and the diversity of organisms already present in the soil (Siciliano and Germida, 1999; Siciliano et al., 2003). Pinton et al. (2001) and Brimecombe, et al. (2001) also summarized that root exudates could change the composition of rhizosphere, and were able to stimulate or inhibit microbial populations and their activities.

However, in the present study, it was difficult to establish if the presence of roots increased or decreased the bacterial diversity because of the poor DGGE image. Use of a higher resolution CCD camera to capture images of the DGGE gels would likely produce higher quality images and thereby allow a more meaningful comparison of the banding patterns and therefore, the microbial populations. As for bacteria group, poor DGGE image of archaea group (Figure 4.12 Lane 3 and 4) made it difficult to draw strong conclusions about the effects of roots on archaea community.

4.4 Conclusions

Enhanced biodegradation of 1,2,3,4-TeCB was observed in the presence of *Typha* roots. Although there were no significant differences in dechlorination pathway between treatments with roots and without roots, dechlorination kinetics increased with increasing amounts of roots, indicating that *Typha* root matter strongly benefited biodegradation of

chlorobenzenes. These results indicate that *Typha*, a native wetland plant with abundant rhizomes, could be a very promising vegetation for application in bioremediation of chlorinated solvents contaminated sites.

Abundance of organic acids, especially acetic acid and propionic acid, and hydrogen were observed in treatments with roots compared to the treatment without roots, which probably caused higher dechlorination activities in root-amended microcosms. Although it is difficult to ascertain the effects of roots on the microbial community from DGGE band profiles, it is most likely that root matter benefited the dechlorinating microorganisms based on the dechlorination kinetics.

CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

Widespread contamination of soil and groundwater by chlorinated benzenes has impacted the environment and public health. Compared to traditional physical/chemical treatments, bioremediation is a promising and cost-effective method, which can prevent the damage of ecological systems since it involves destruction of pollutants by natural mechanisms. Therefore, it is important to investigate the mechanisms involved in attenuation of pollutants and the factors affecting them before a decision of applying bioremediation for cleaning up a contaminated site is reached. Microcosm studies were, therefore, undertaken to investigate the potential of 1,2,3,4-Tetrachlorobenzene (1,2,3,4-TeCB) biodegradation in one mineral-dominated soil: PPI (Petro Processors Inc. site) soil, and three organic matter-dominated soils (natural wetland soil, constructed wetland soil (a mixture of peat, compost and sand), and river sediment). Concentrations of hydrogen and methane associated with dechlorination activities were also measured. To determine whether methanogens were directly responsible for dechlorination, 2-bromoethanesulfonic acid (BES) was used for inhibition of methanogenesis. Microbial diversity of dechlorinating populations was also analyzed by using the PCR-DGGE technique. To better understand the factors affecting dechlorination activities, the present study involved three generation cultures. The 1st Generation culture was developed from the first spike of the test chemical into the fresh soil, whereas the 2nd and 3rd generation cultures were prepared by inoculating 25 mL of slurries from the immediately previous culture.

Phytoremediation, a relatively new bioremediation technology using plants and associated rhizosphere microorganisms, has shown great potential of enhancing

biodegradation. Organic acids from carbonaceous root matter may serve as direct electron donors or precursors of hydrogen, necessary for driving dechlorination reactions, and thus enhance the biodegradation activities. Increased microbial population and diversity due to the presence of plant roots may also contribute to the enhanced biodegradation activities. To investigate the effects of wetland plant roots on anaerobic dechlorination of 1,2,3,4-TeCB, comparative studies on degradation kinetics in the presence and absence of *Typha latifolia L*. roots were conducted. Microbial diversity using PCR based DGGE technique, organic acids, hydrogen and methane were also measured to better understand the effects of *Typha* roots on dechlorination of chlorobenzenes.

Results of the present study have shown that 1,2,3,4-TeCB was able to be completely degraded in all organic matter- and mineral-dominated soils under anaerobic conditions. The most dominant dechlorination pathway of 1,2,3,4-TeCB was: 1,2,3,4-TeCB → 1,2,3-TCB → 1,2-DCB + 1,4-DCB + 1,3-DCB → monochlorobenezene + benzene. The test chemical was biodegraded at rates ranging from 0.023 day^{-1} (half-life time of 30.5 days) to 1.108 day^{-1} (half-life time of 0.6 days), with lag periods varied between 1 and 72 days. There was no apparent relationship between dechlorination rate of the test chemical and organic carbon content of the test soils. Besides organic carbon content, dechlorination kinetics of chlorobenzenes depends on other factors such as previous exposure history. DGGE banding profiles suggested that different microbial communities were involved in biodegradation of 1,2,3,4-TeCB. Higher dechlorination kinetic rate and shorter lag period can be expected with increasing number of inoculations, if soil characteristics and experimental conditions remain unchanged. Moreover, complete dechlorination of the parent compound and intermediate daughter

products (trichlorobenzenes and dichlorobenzenes) were observed in the 3rd Generation cultures of active control microcosms of all test soils. In addition, constructed wetland soil was found to be a very promising material for *ex-situ* bioremediation of chlorinated solvents-contaminated sites using treatment wetland systems. Moreover, natural wetland soil was found to have intrinsic capability of attenuating chlorinated benzenes.

Generally, there was no clear relationship between hydrogen concentrations and methanogenesis or dechlorination. However, hydrogen concentration trends in some treatments suggested that hydrogen was probably used as an electron donor during methanogenesis and for driving dechlorination reactions. Methane started to accumulate after 1,2,3,4-TeCB and its intermediate daughter products (trichlorobenzenes and dichlorobenzenes) were completely degraded in most active control microcosms, indicating that dechlorinators may have out-competed methanogens for electron donors.

Enhanced biodegradation of 1,2,3,4-TeCB was observed in the presence of *Typha latifolia L.* roots, and biodegradation rate increased with increasing ratio of roots to soil. Therefore, *Typha*, a native wetland plant with abundant rhizomes, could be a very promising vegetation for application in bioremediation of chlorinated solvents-contaminated sites. Higher dechlorination activities in root-amended microcosms were probably caused by higher concentrations of organic acids especially acetic acid and propionic acid, and consequently hydrogen in treatments with roots compared to the treatment without roots due to carbonaceous root matter. DGGE banding profiles revealed that the presence of roots changed the bacterial community, but it is difficult to ascertain whether the bacteria diversity increased due to the root matter because of the poor image. In view of this, improvement of the DGGE imaging system is recommended.

It was difficult to assure that bacteria but not methanogens were directly responsible for dechlorination by using BES for inhibition of methanogenesis. It may be clearer by tracking the changes in microbial composition and diversity of both bacteria and archaea with time in both active control and BES-amended treatments. In addition, specific primers for methanogens are recommended instead of using archaea-specific primers. Similarly, to understand more about the effects of roots on microbial community and thus on dechlorination, temporal monitoring of microbial population and diversity in treatments with different amount of roots and without roots is recommended.

The limitation of using excised roots is that it was difficult to determine the relative contribution of root exudates and decomposition of roots themselves in organic acids production and consequently hydrogen concentrations, since organic acids can be produced from both root secretions and decomposition of the roots themselves. To ascertain that it was root exudates but not decomposition of roots that enhanced dechlorination of chlorobenzenes, it is recommended to collect root exudates from the live plant and use them for microcosm study to investigate their effects on dechlorination.

Before field application, further investigations, such as mesocosm and pilot scale studies using live plants, on the potential of *Typha* to enhance reductive dechlorination of chlorobenzenes are recommended.

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APPENDIX

APPENDIX I: DECHLORINATION PROFILES AND METHANE AND HYDROGEN CONCENTRATION TRENDS IN NATURAL WETLAND SOIL

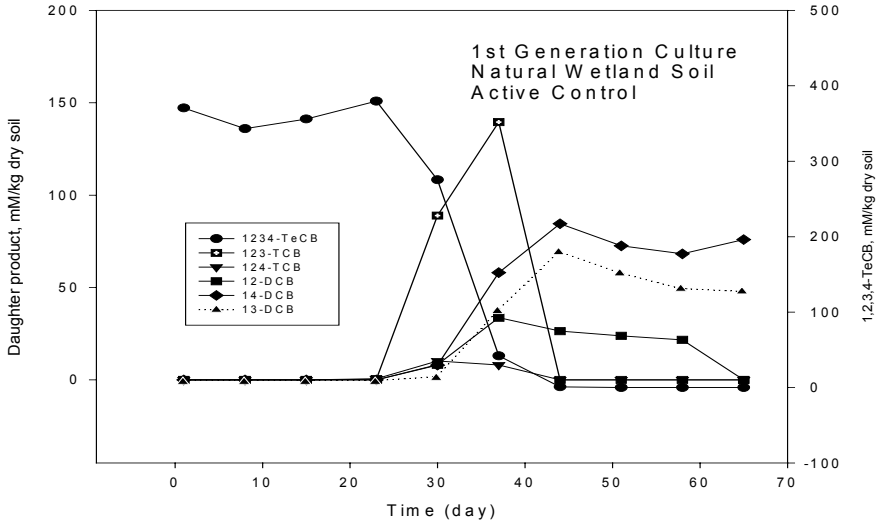

Figure A1: Dechlorination profile of the 1st Generation culture of active control natural wetland soil microcosms.

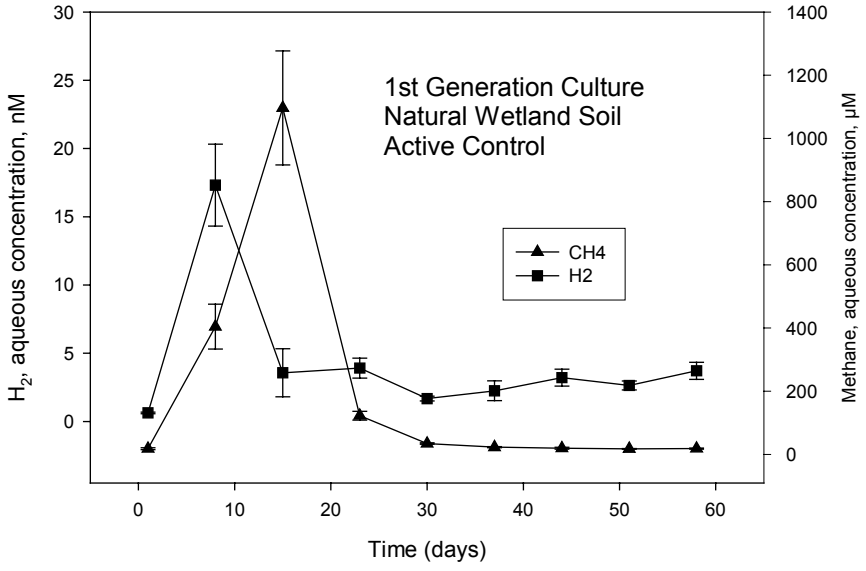

Figure B1: Methane and hydrogen concentrations of the 1st Generation culture of active control natural wetland soil microcosms.

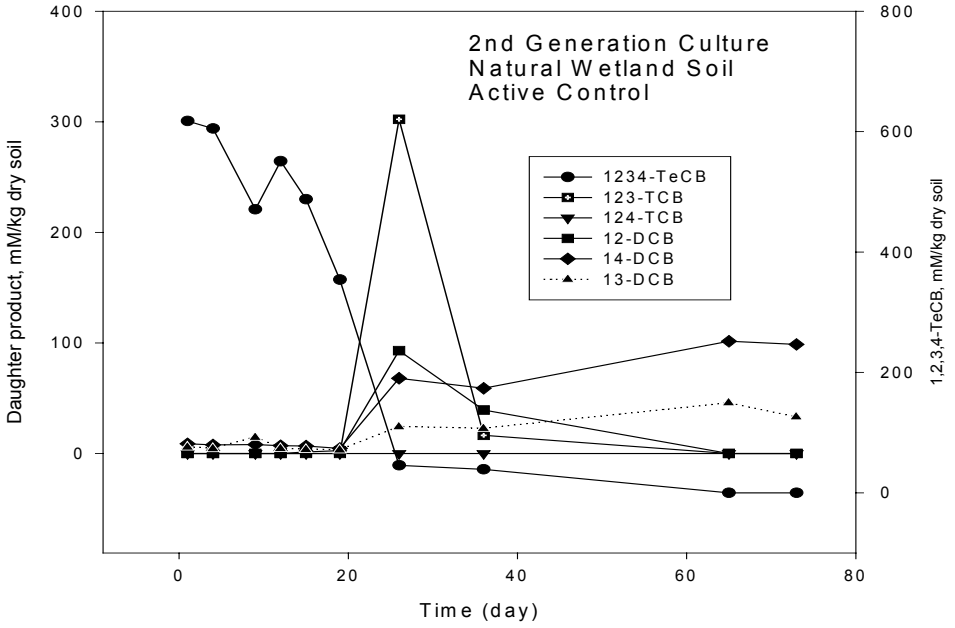

Figure A2: Dechlorination profile of the 2nd Generation culture of active control natural wetland soil microcosms.

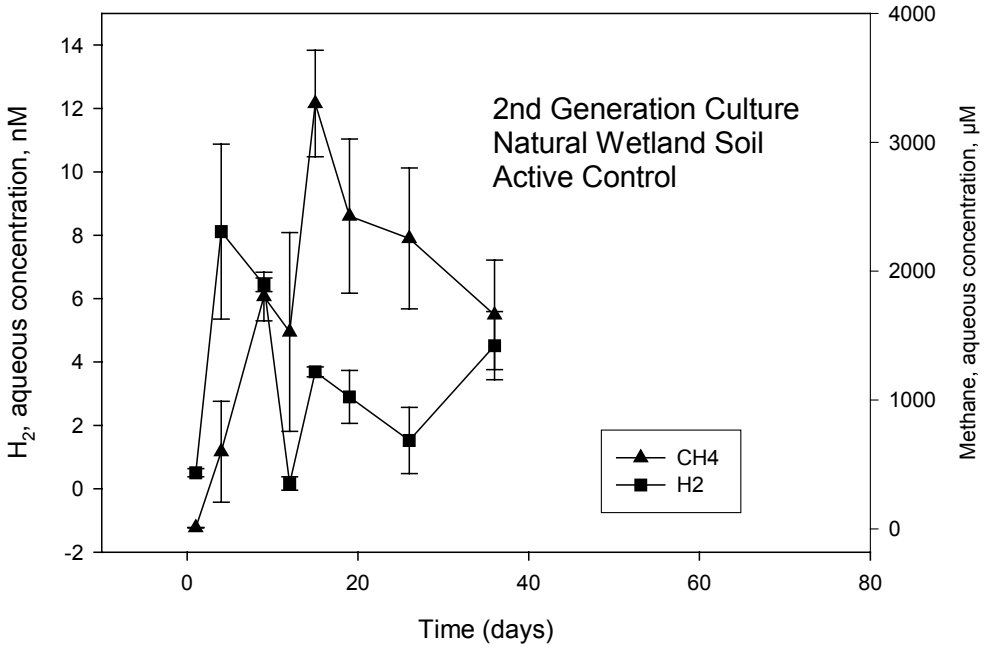

Figure B2: Methane and hydrogen concentrations of the 2nd Generation culture of active control natural wetland soil microcosms

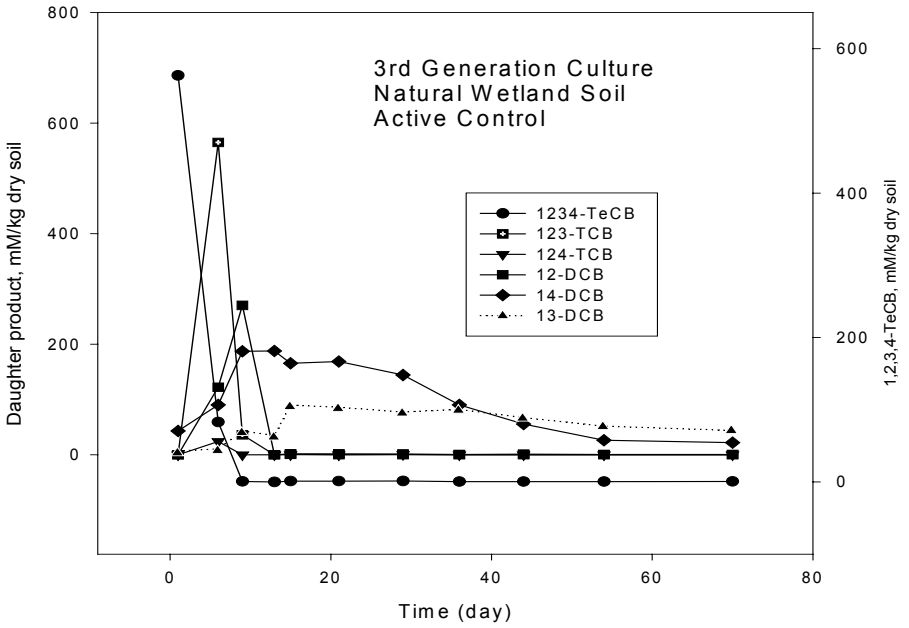

Figure A3: Dechlorination profile of the 3rd Generation culture of active control natural wetland soil microcosms.

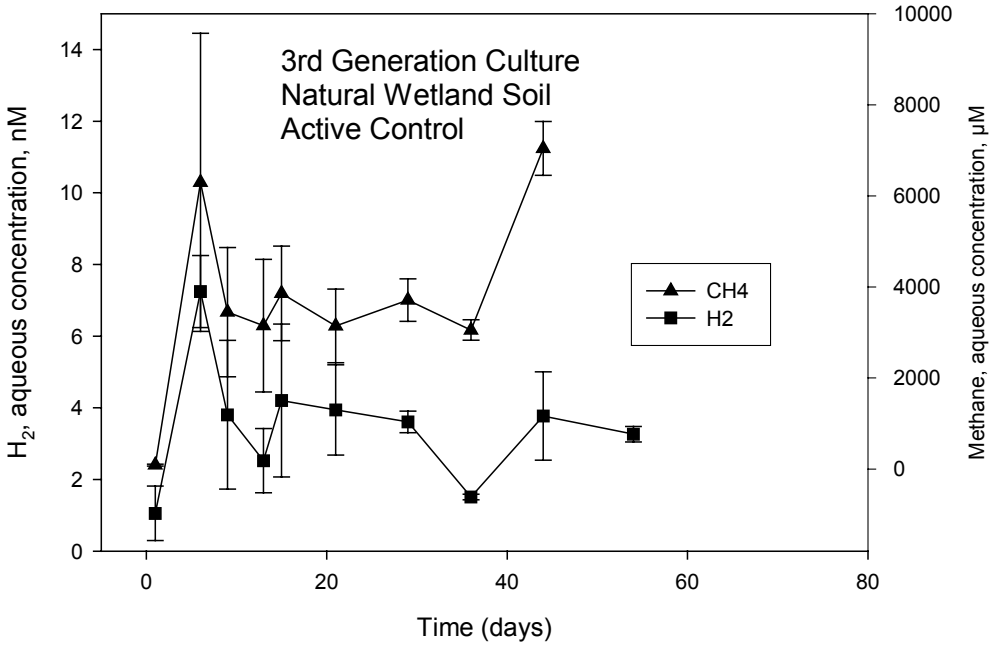

Figure B3: Methane and hydrogen concentrations of the 3rd Generation culture of active control natural wetland soil microcosms.

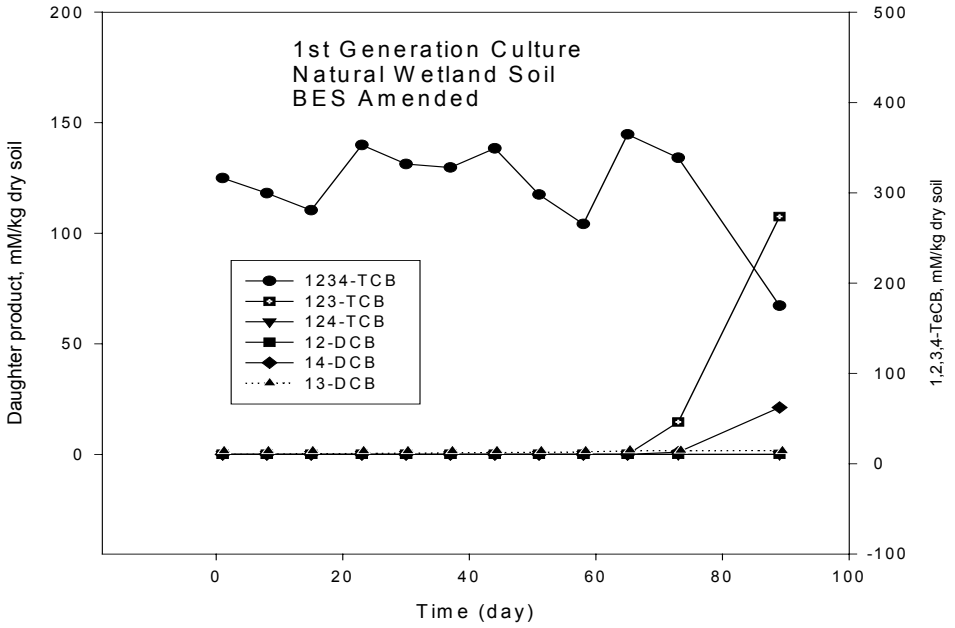

Figure A4: Dechlorination profile of the 1st Generation culture of BES-amended natural wetland soil microcosms.

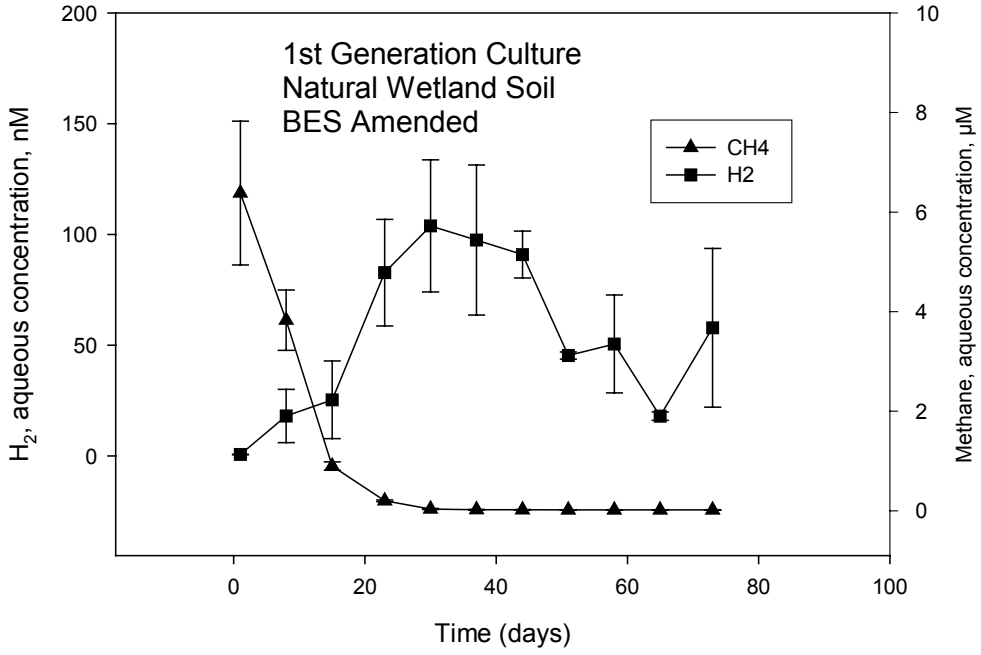

Figure B4: Methane and hydrogen concentrations of the 1st Generation culture of BES-amended natural wetland soil microcosms.

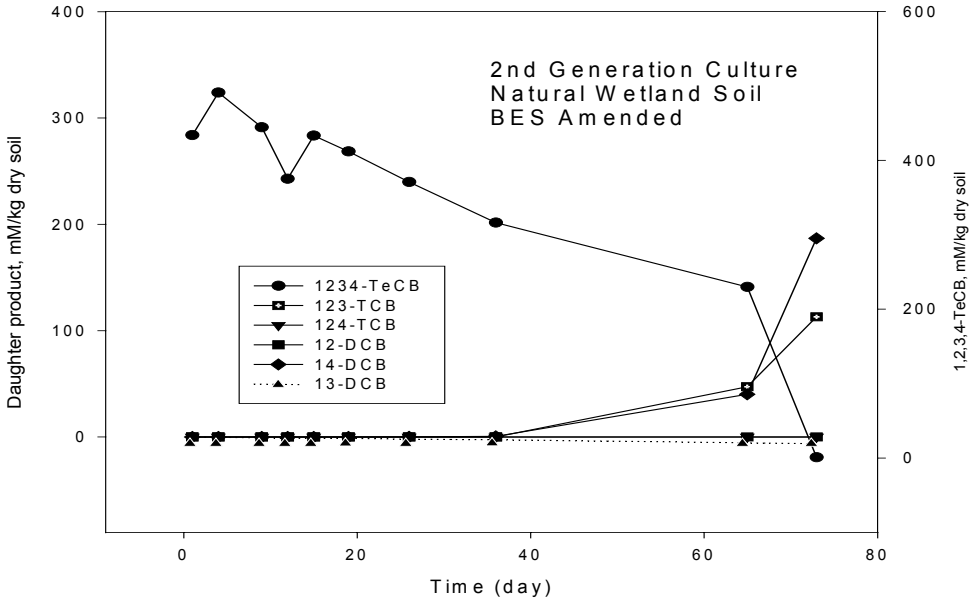

Figure A5: Dechlorination profile of the 2nd Generation culture of BES-amended natural wetland soil microcosms.

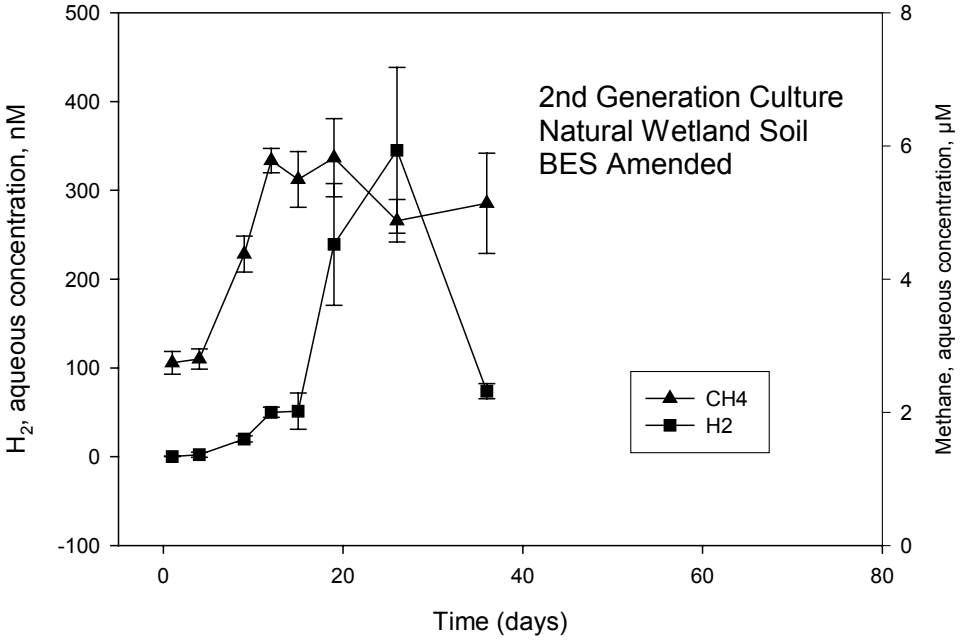

Figure B5: Methane and hydrogen concentrations of the 2nd Generation culture of BES-amended natural wetland soil microcosms.

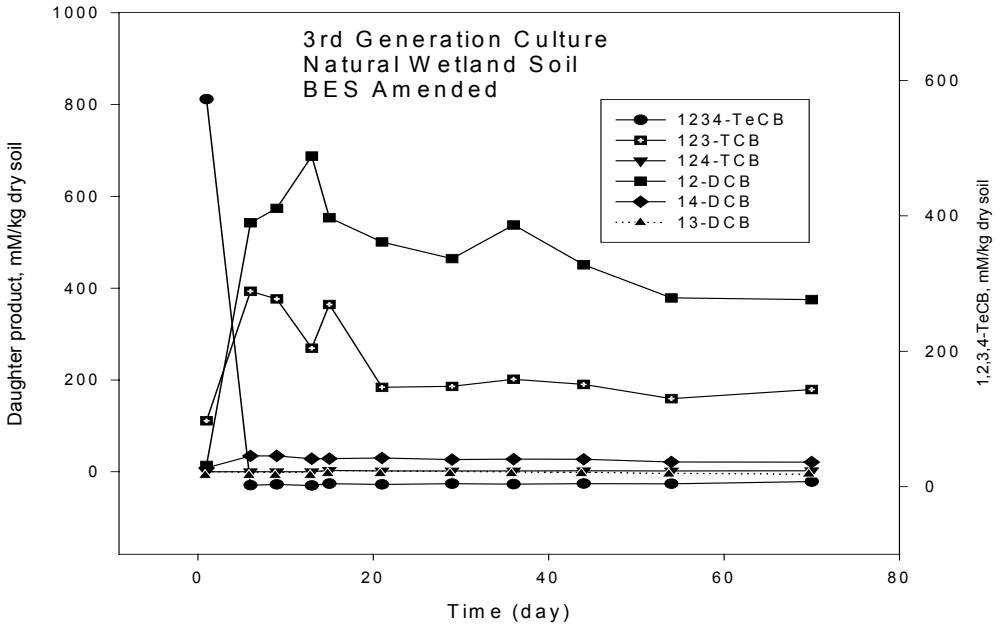

Figure A6: Dechlorination profile of the 3rd Generation culture of BES-amended natural wetland soil microcosms.

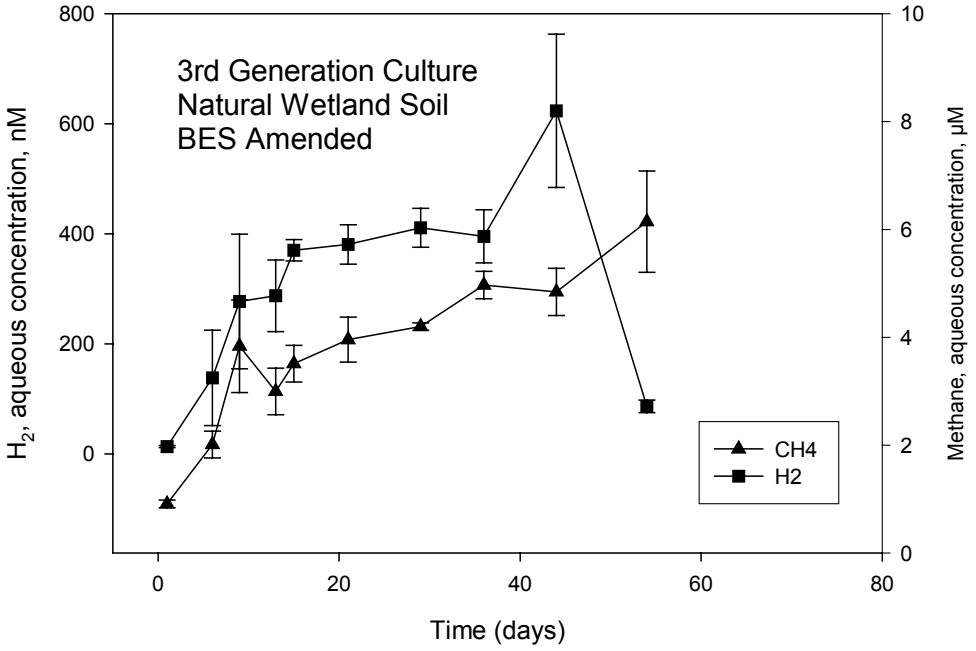

Figure B6: Methane and hydrogen concentrations of the 3rd Generation culture of BES-amended natural wetland soil microcosms.

APPENDIX II: DECHLORINATION PROFILES AND METHANE AND HYDROGEN CONCENTRATION TRENDS IN RIVER SEDIMENT

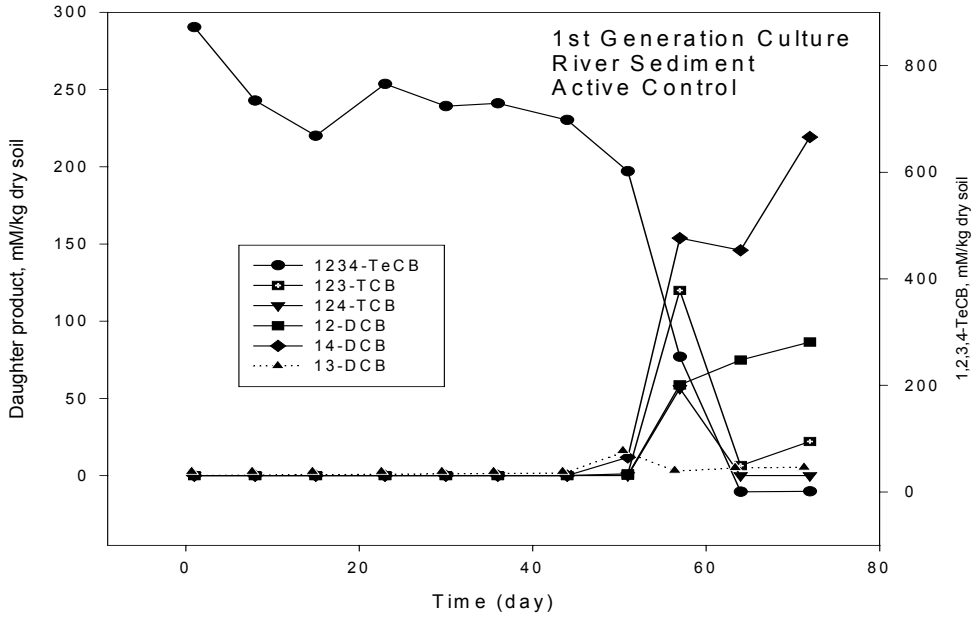

Figure C1: Dechlorination profile of the 1st Generation culture of active control river sediment microcosms.

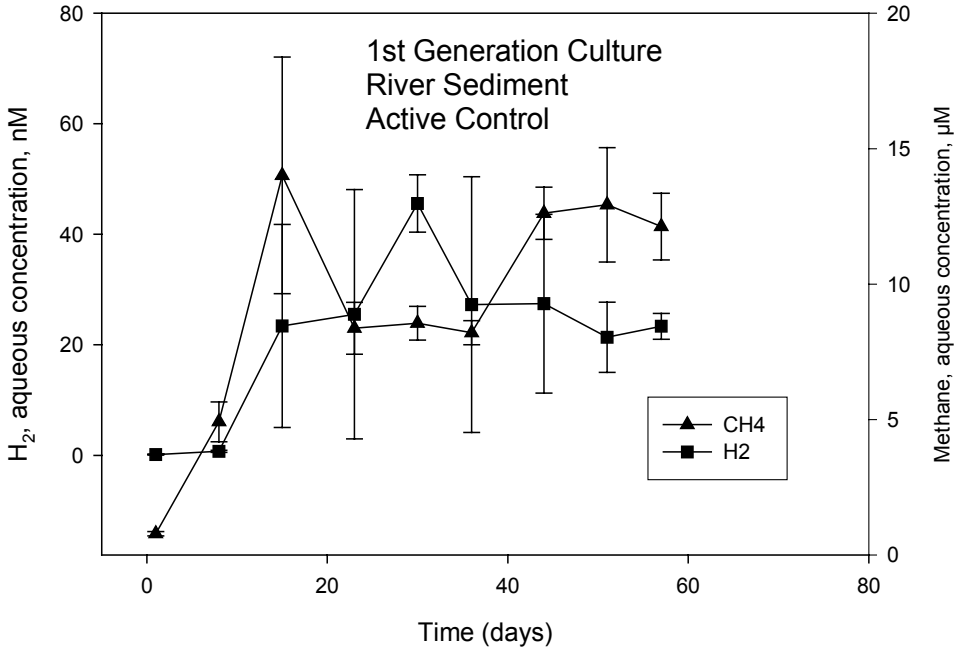

Figure D1: Methane and hydrogen concentrations of the 1st Generation culture of active control river sediment microcosms.

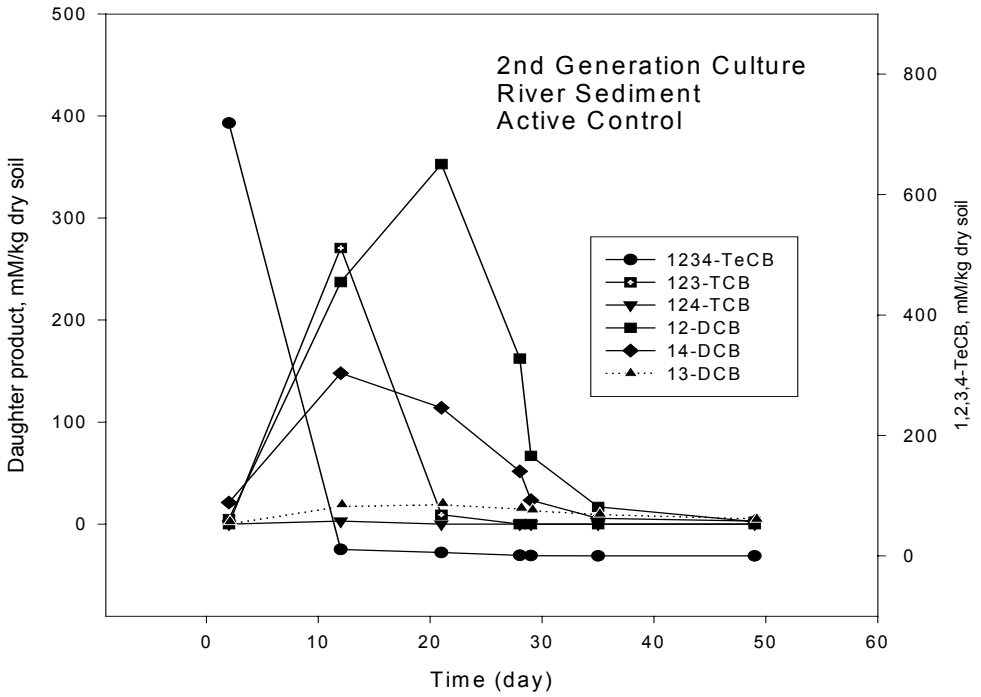

Figure C2: Dechlorination profile of the 2nd Generation culture of active control river sediment microcosms.

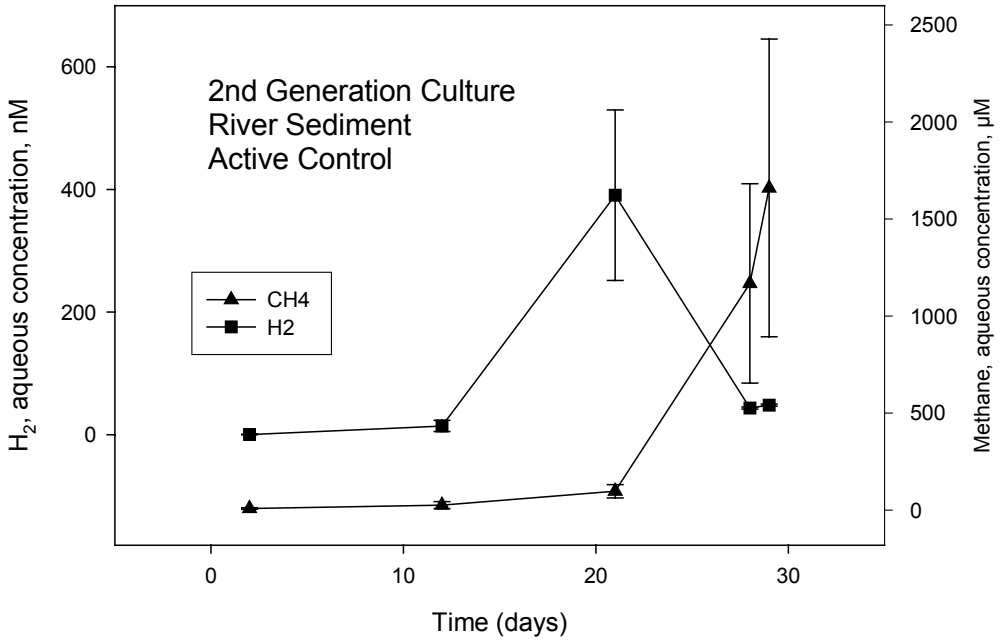

Figure D2: Methane and hydrogen concentrations of the 2nd Generation culture of active control river sediment microcosms.

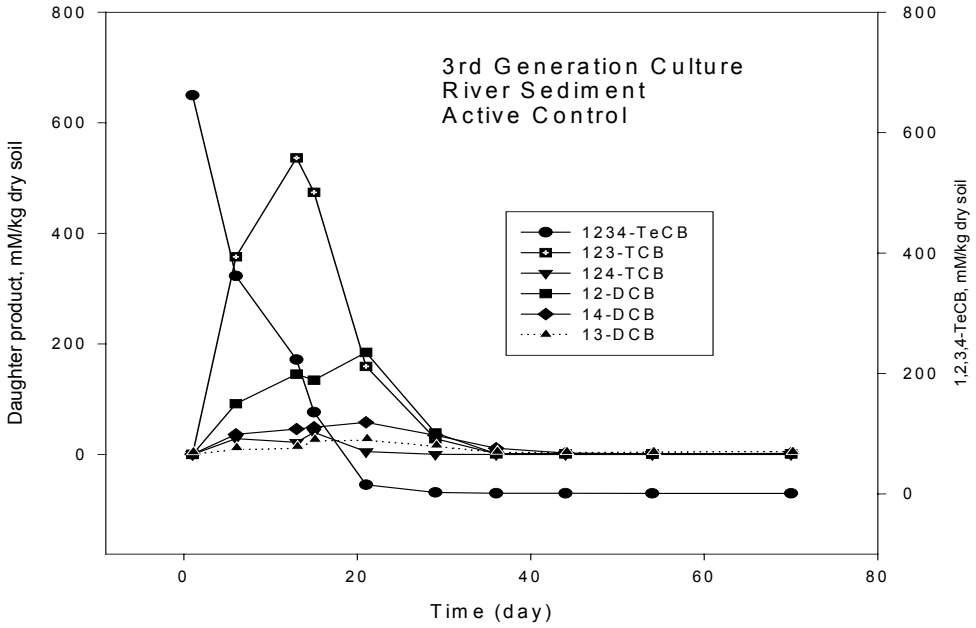

Figure C3: Dechlorination profile of the 3rd Generation culture of active control river sediment microcosms.

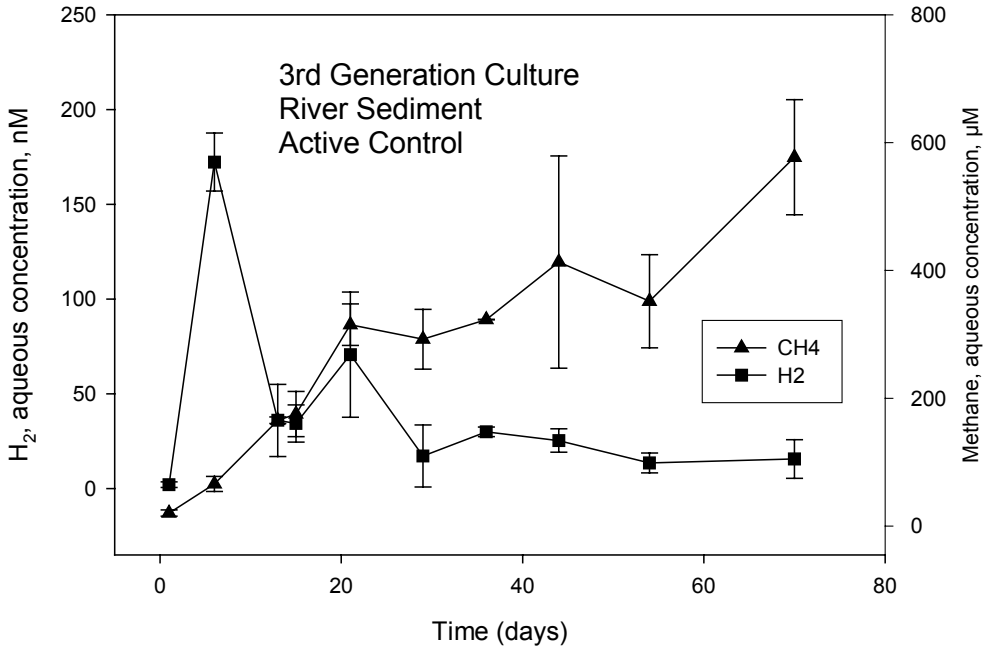

Figure D3: Methane and hydrogen concentrations of the 3rd Generation culture of active control river sediment microcosms.

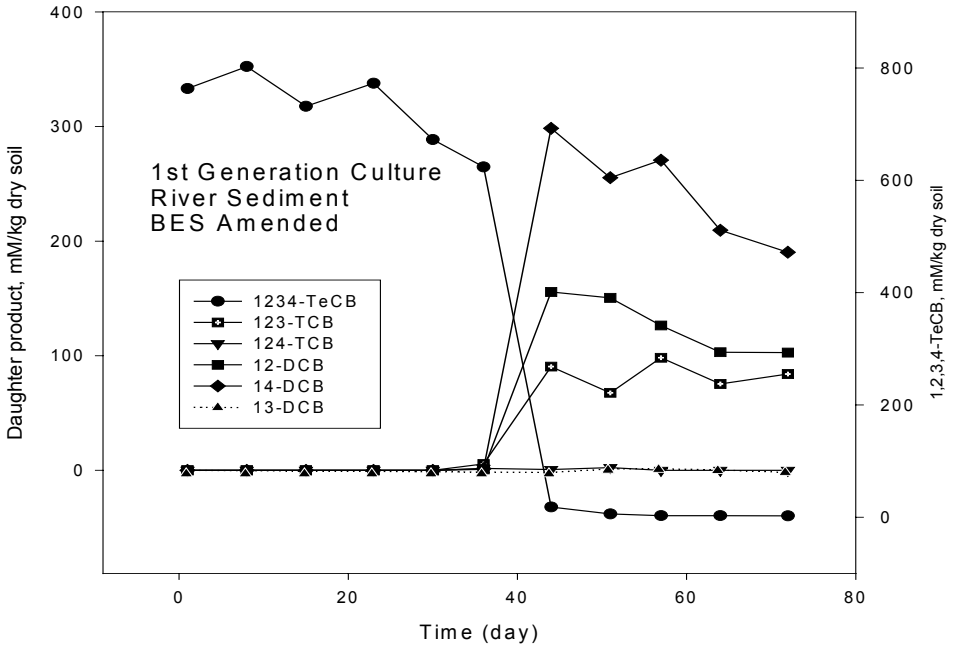

Figure C4: Dechlorination profile of the 1st Generation culture of BES-amended river sediment microcosms.

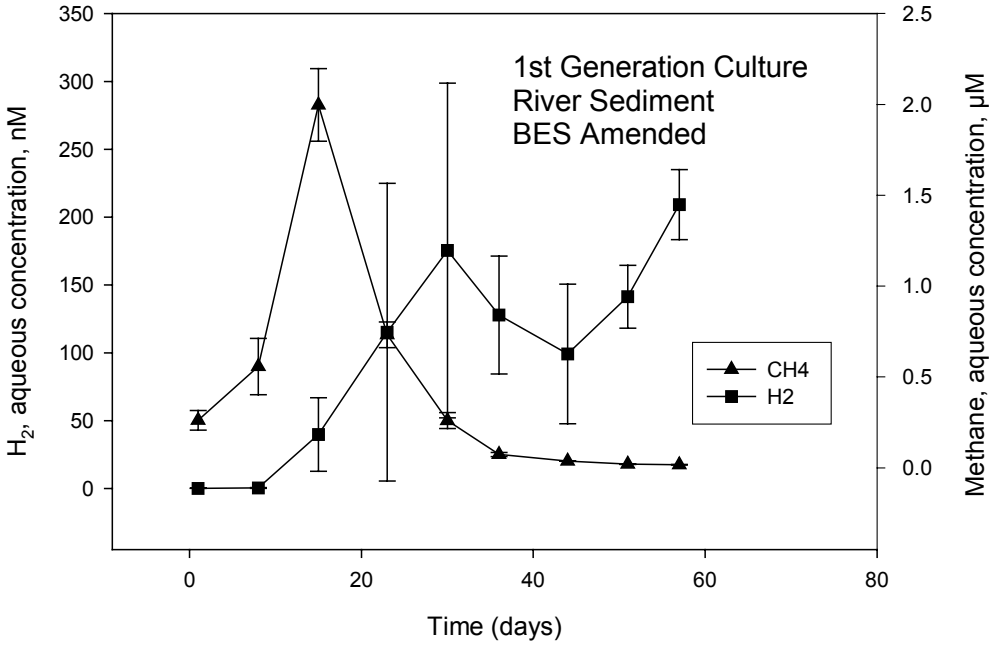

Figure D4: Methane and hydrogen concentrations of the 1st Generation culture of BES-amended river sediment microcosms.

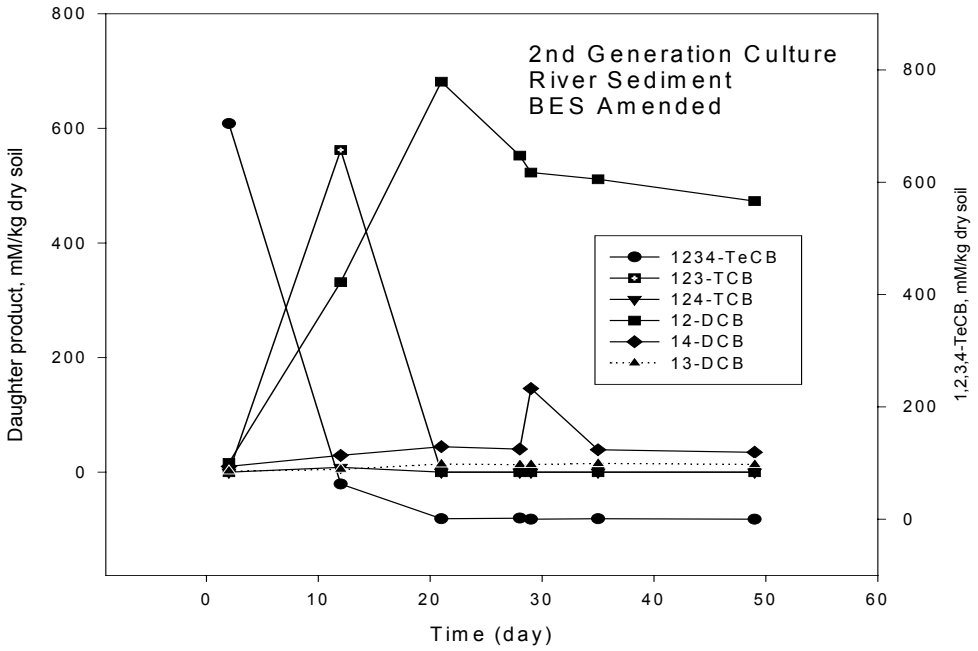

Figure C5: Dechlorination profile of the 2nd Generation culture of BES-amended river sediment microcosms.

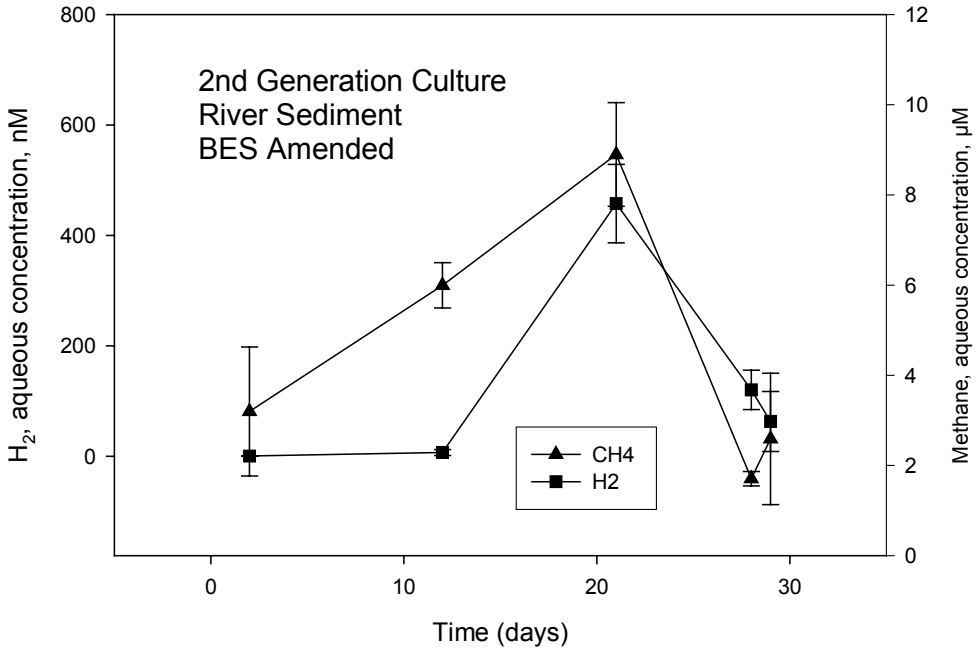

Figure D5: Methane and hydrogen concentrations of the 2nd Generation culture of BES-amended river sediment microcosms.

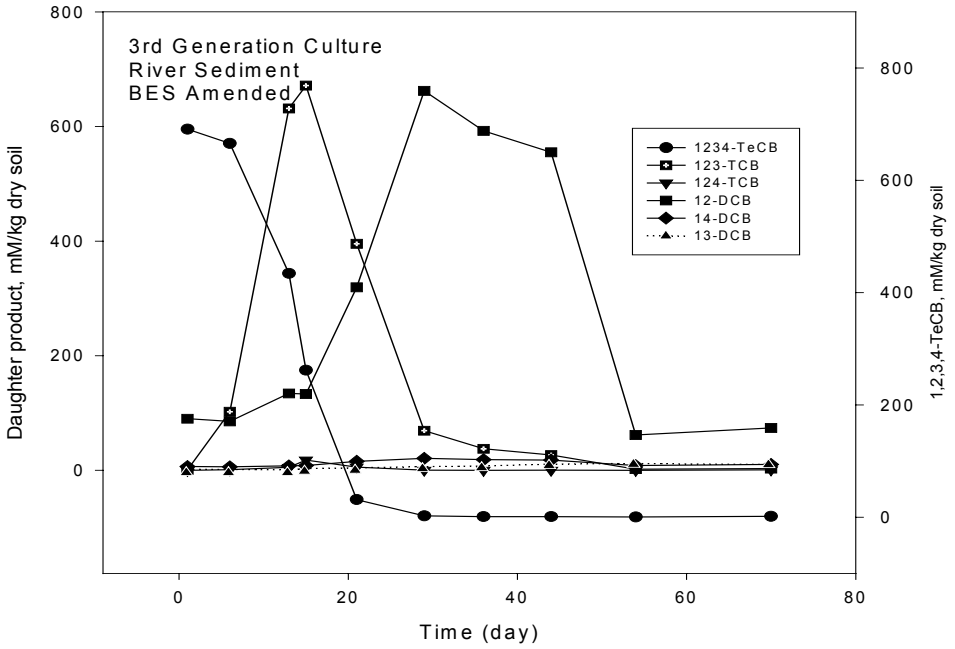

Figure C6: Dechlorination profile of the 3rd Generation culture of BES-amended river sediment microcosms.

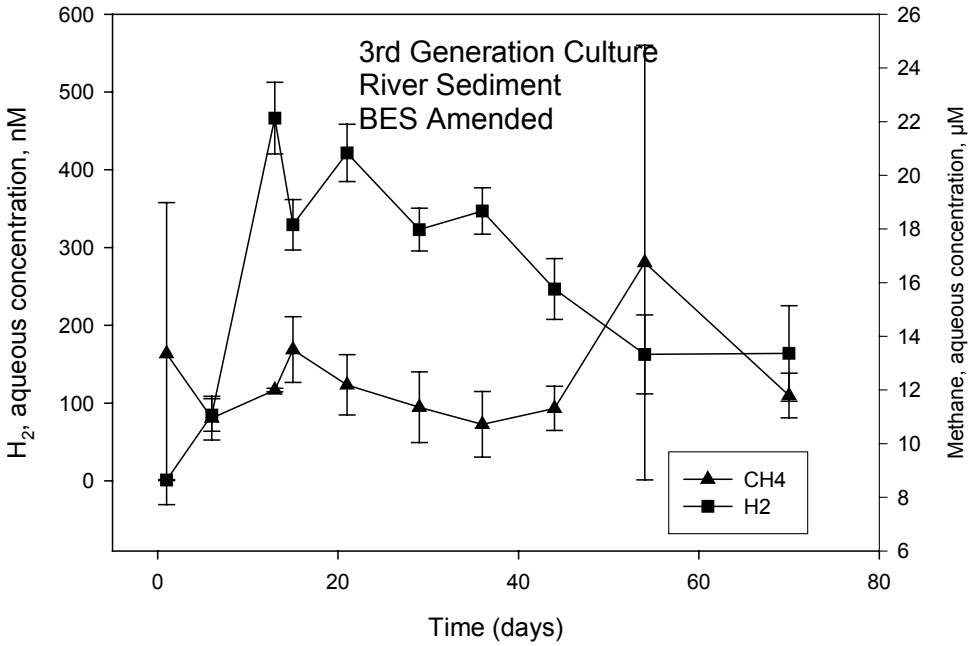

Figure D6: Methane and hydrogen concentrations of the 3rd Generation culture of BES-amended river sediment microcosms

APPENDIX III: DECHLORINATION PROFILES AND METHANE AND HYDROGEN CONCENTRATION TRENDS IN PPI SOIL

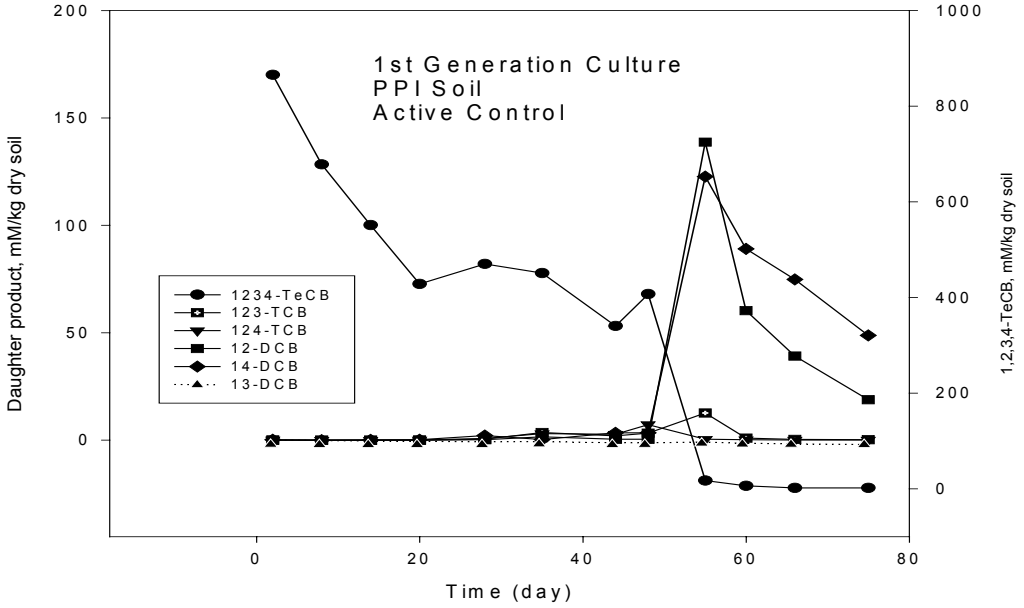

Figure E1: Dechlorination profile of the 1st Generation culture of active control PPI soil microcosms.

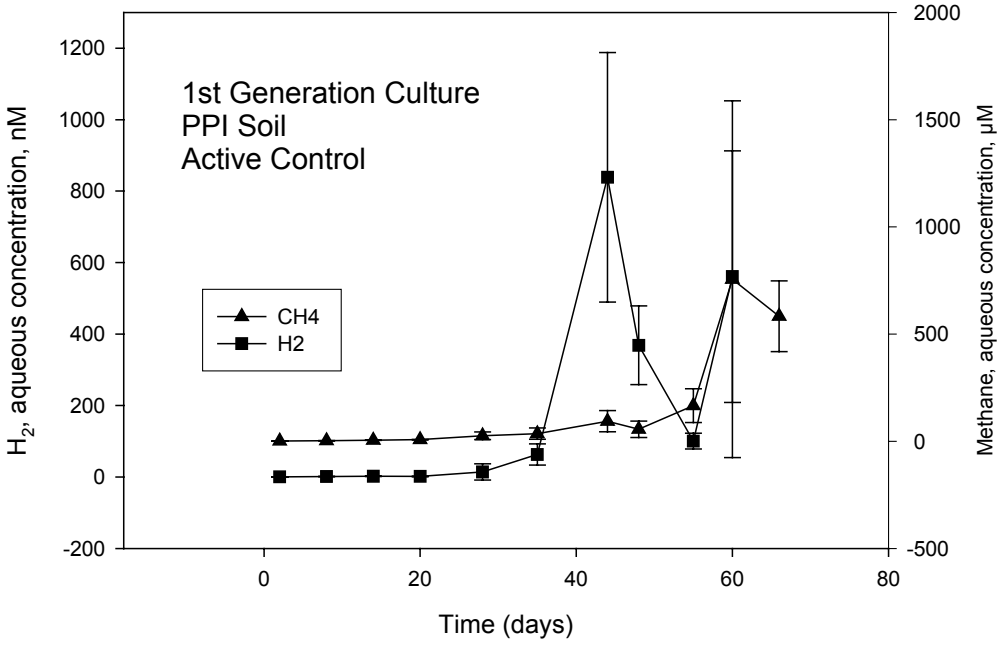

Figure F1: Methane and hydrogen concentrations of the 1st Generation culture of active control PPI soil microcosms.

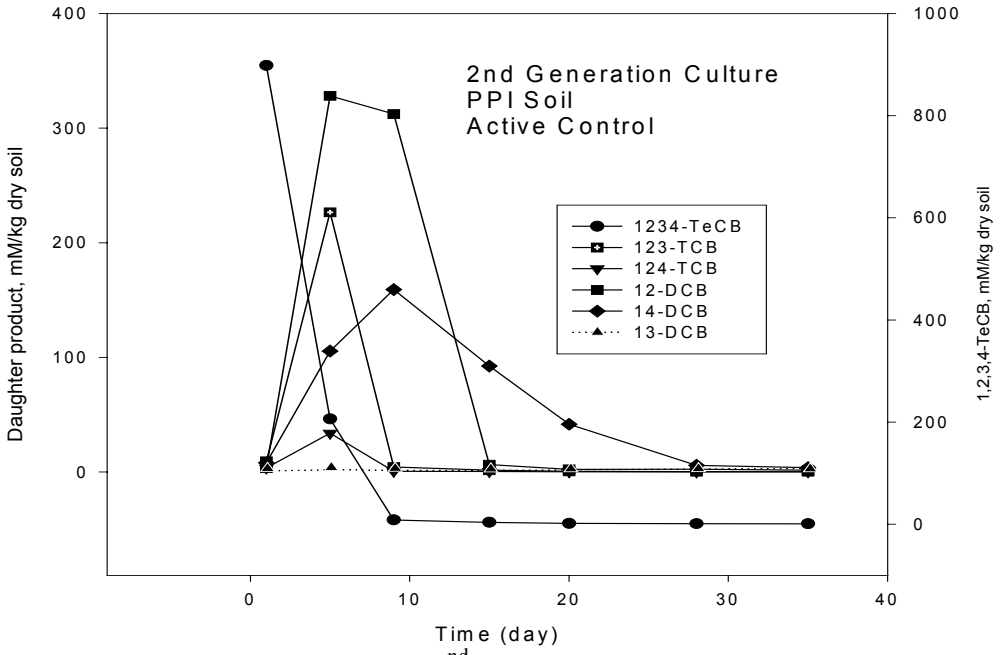

Figure E2: Dechlorination profile of the 2nd Generation culture of active control PPI soil microcosms.

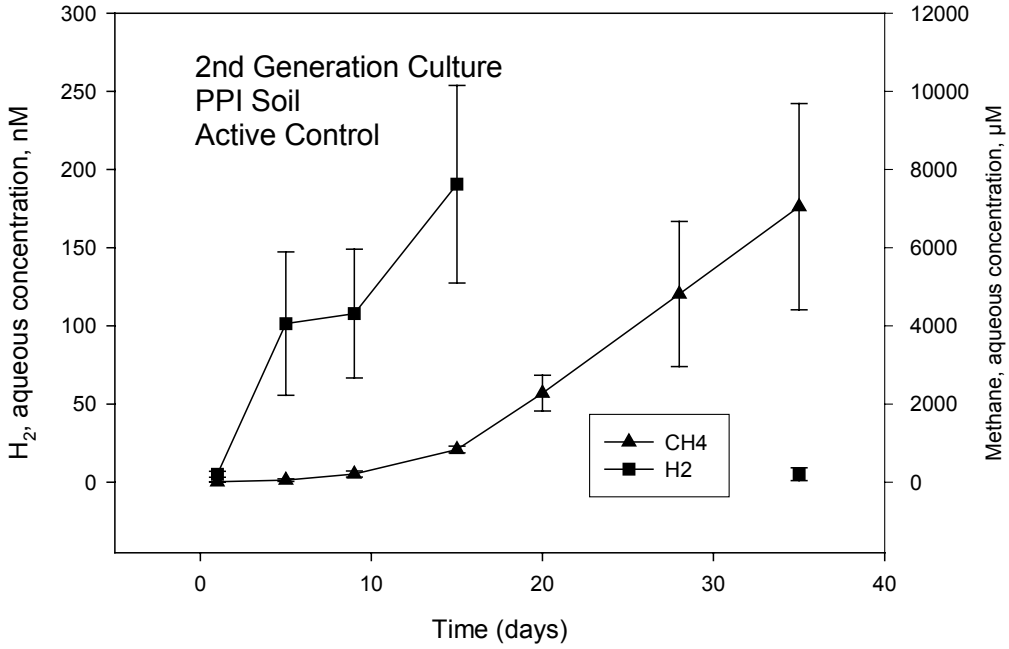

Figure F2: Methane and hydrogen concentrations of the 2nd Generation culture of active control PPI soil microcosms.

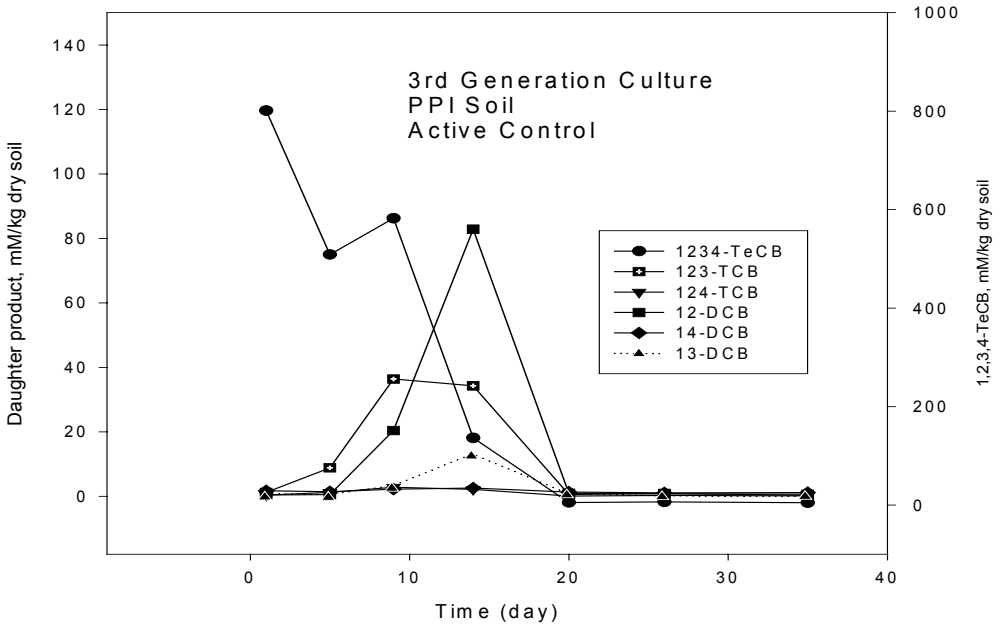

Figure E3: Dechlorination profile of the 3rd Generation culture of active control PPI soil microcosms.

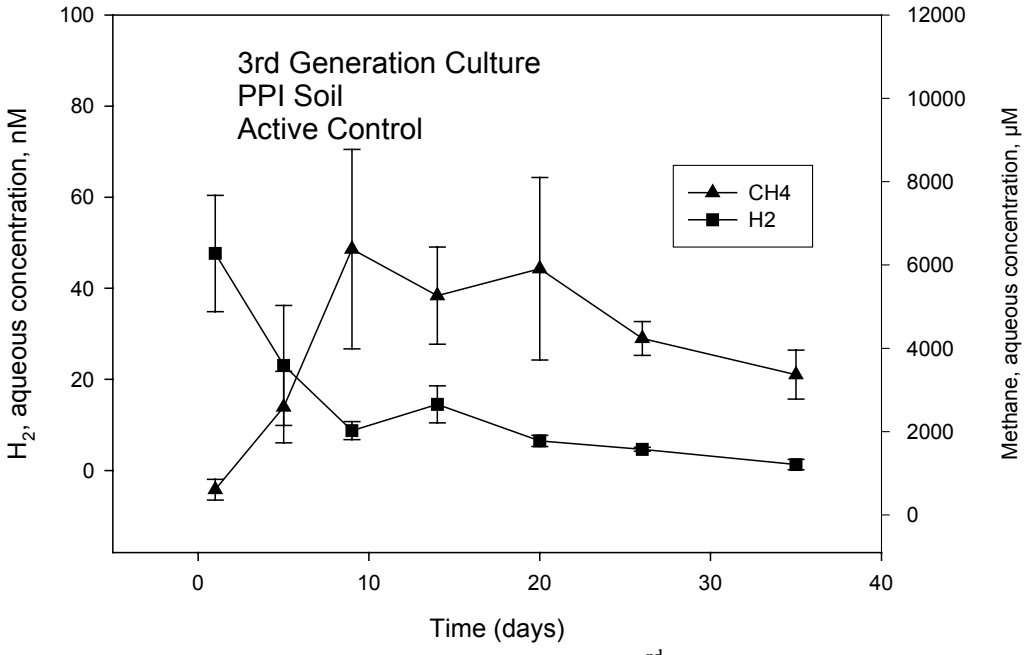

Figure F3: Methane and hydrogen concentrations of the 3rd Generation culture of active control PPI soil microcosms.

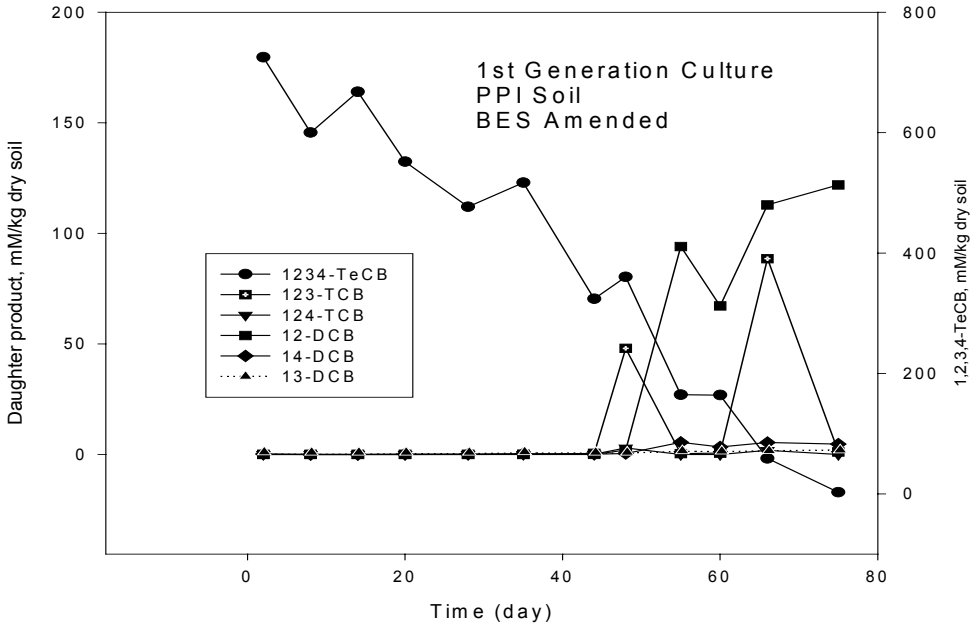

Figure E4: Dechlorination profile of the 1st Generation culture of BES-amended PPI soil microcosms

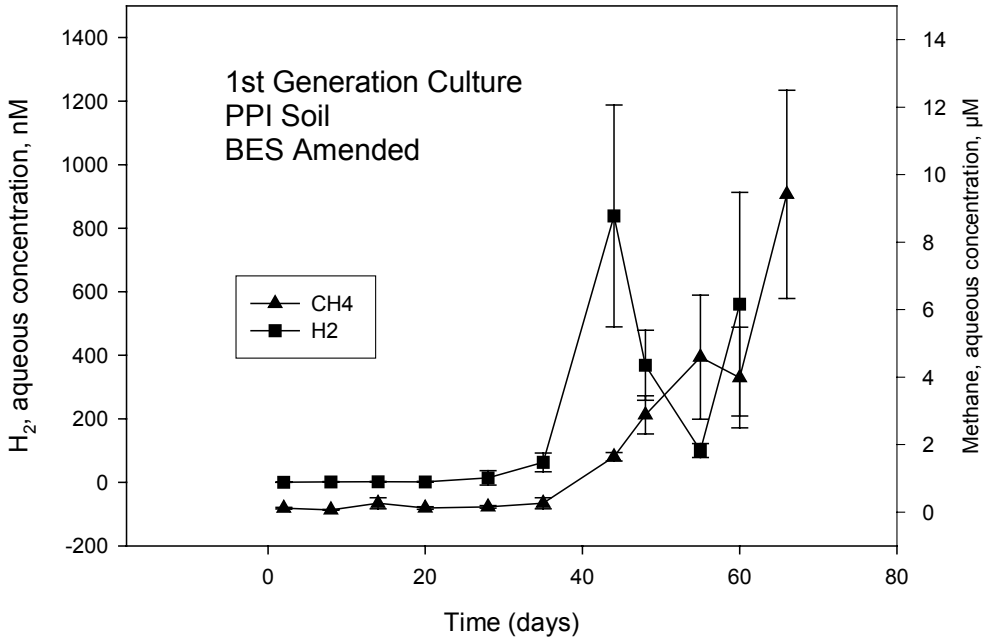

Figure F4: Methane and hydrogen concentrations of the 1st Generation culture of BES-amended PPI soil microcosms

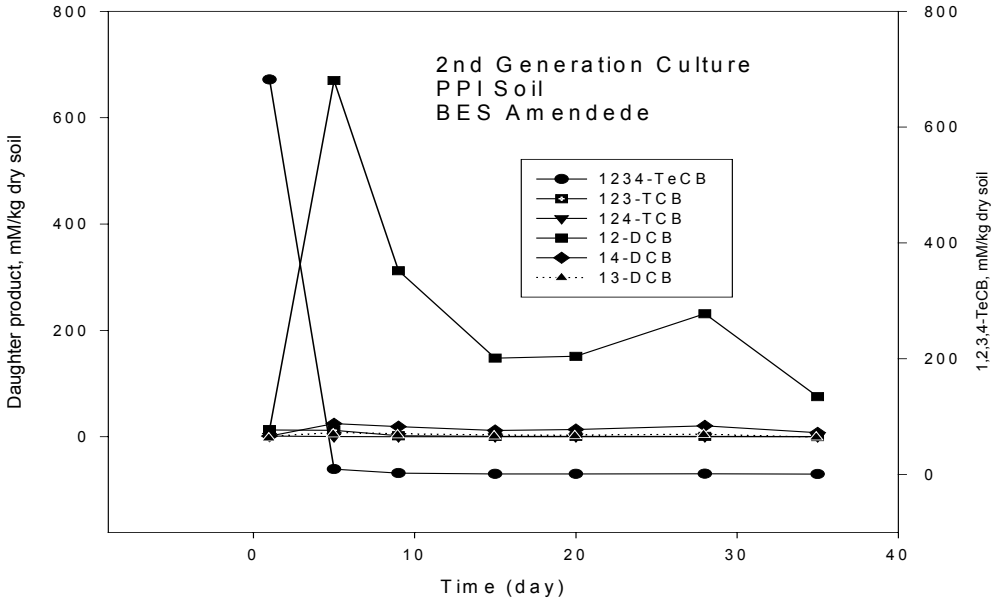

Figure E5: Dechlorination profile of the 2nd Generation culture of BES-amended PPI soil microcosms.

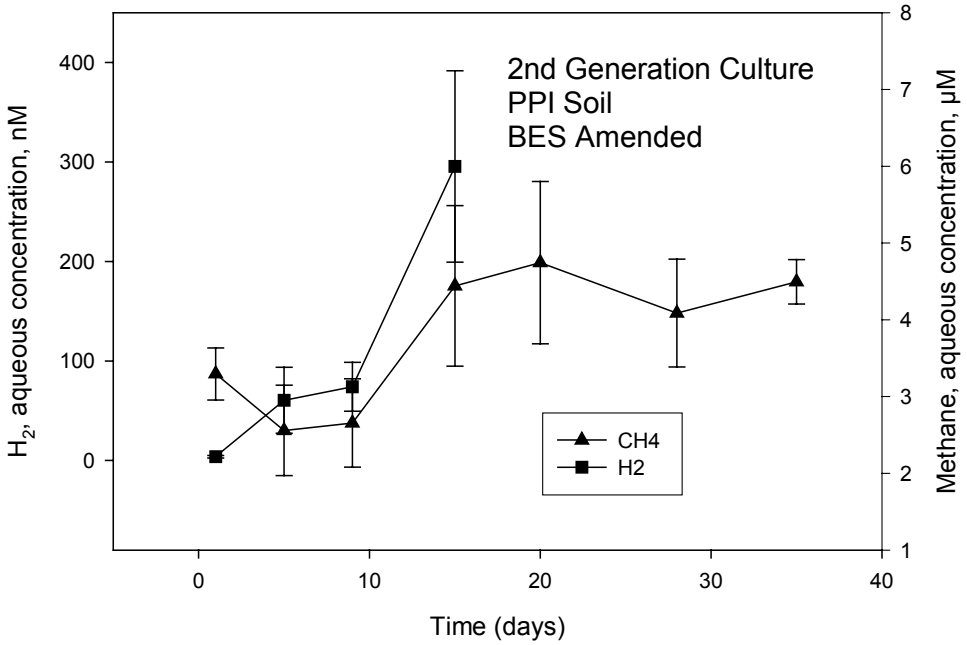

Figure F5: Methane and hydrogen concentrations of the 2nd Generation culture of BES-amended PPI soil microcosms.

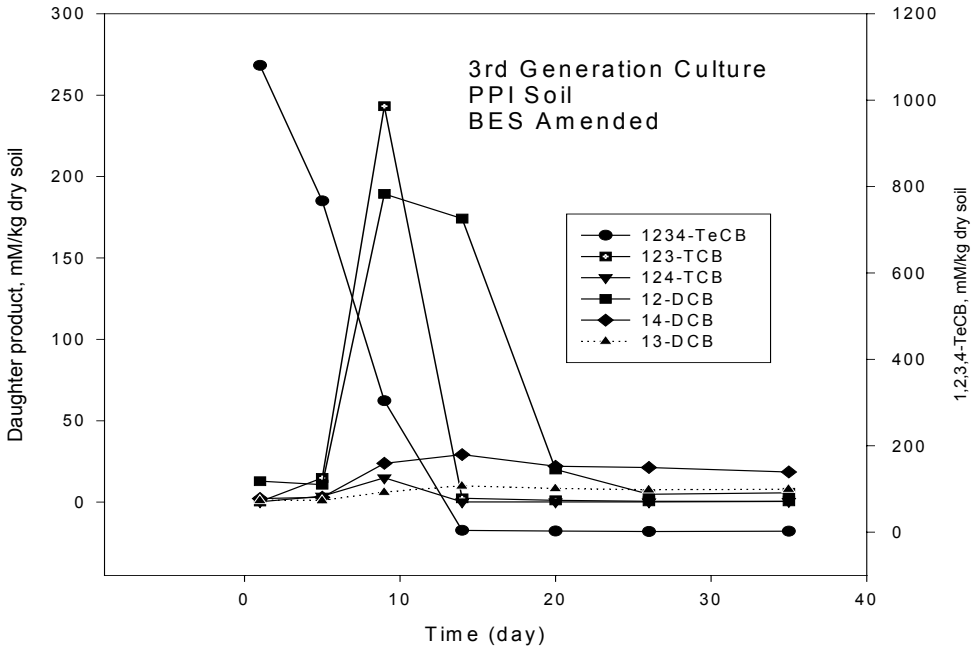

Figure E6: Dechlorination profile of the 3rd Generation culture of BES-amended PPI soil microcosms.

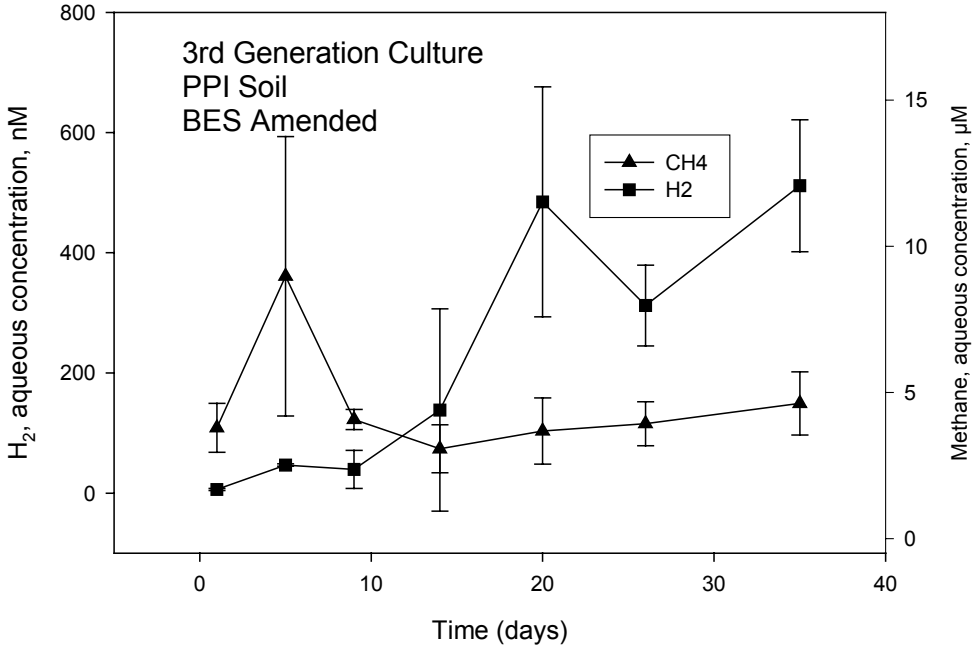

Figure F6: Methane and hydrogen concentrations of the 3rd Generation culture of BES-amended PPI soil microcosms.

VITA

Lizhu Lin was born in February 9, 1978, in Minhou, Fujian, China. She graduated from Xiamen University in Xiamen, Fujian, China, in July 2000, with a Bachelor of Science degree in chemical engineering.

In January 2001, she enrolled in the graduate program in civil engineering at Louisiana State University in Baton Rouge, Louisiana. She will graduate in May 2003, with a Master of Science in Civil Engineering degree, majoring in environmental engineering. She is planning to pursue a doctoral program in environmental engineering after graduation.